UNIVERSITY OF CALIFORNIA

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Investigating the role of age and sex on the affective and cognitive consequences of alcohol binge-drinking

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Psychological & Brain Sciences

by

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Abstract

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This dissertation investigates how age of drinking onset and biological sex influence the affective and cognitive consequences that follow from a history of alcohol binge-drinking. Using a rodent model of voluntary binge-drinking, this research examines how a history of binge-drinking impacts withdrawal-induced negative affect, spatial and working memory, and protein expression through its effects on key brain regions involved in these processes. The findings suggest that binge-drinking during adolescence leads both to long-term cognitive deficits and increases in anxiety and depression-like behaviors in adulthood. As in the human population, female rodents in the present data exhibit earlier and more severe behavioral disturbances during alcohol withdrawal relative to males—underlining the urgent need for sex-specific approaches in managing alcohol use disorder. Additionally, this research explores how sex-specific chemosensory stimuli influence anxiety-related behaviors, providing insights into environmental and procedural factors modulating the manifestation of negative affect. A key finding includes the identification of sex-dependent variations in the modulation of marble-burying behavior in response to sex-related odors. Biochemical analyses reveal significant alterations in glutamate receptor expression and other neuropathological markers in the prefrontal cortex, entorhinal cortex, and amygdala, which may be implicated in the animals' observed behavioral anomalies. These results

suggest that early alcohol exposure disrupts normal brain development, resulting in long-lasting effects that may manifest in later life. Taken together, this research offers key insights into the complex effects of age and sex on the neurobiological and behavioral impacts of alcohol binge-drinking. More broadly, these results highlight the benefits of creating distinct interventions across age groups and sexes both for treating alcohol use disorder and managing its cognitive and affective consequences.

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General Introduction

1.1 Introduction

Alcohol's longstanding role in the cultural fabric of communities around the world embodies an interesting paradox, both as a celebrated agent of social bonding and as an addictive psychoactive substance. This inherent duality highlights alcohol's rank as the most abused psychoactive substance globally, despite considerable evidence surrounding its adverse effects (Griswold et al., 2018; SAMHSA, 2021; WHO, 2018). Consequently, the global impact of alcohol misuse is significant, with alcohol-related factors contributing to an astounding death rate of 1 in every 20 deaths (WHO, 2018). Furthermore, according to the World Health Organization (WHO), the harmful use of alcohol results in over 200 diseases and injuries, with an estimated 3 million deaths a year, which accounts for 5.3% of all deaths worldwide (WHO, 2022).

Within this context of widespread alcohol consumption, binge-drinking emerges as a particularly alarming and destructive pattern of intake. Characterized by the rapid and excessive consumption of alcohol within a 2-hour period, binge-drinking is defined as a pattern of alcohol consumption that raises blood alcohol concentrations (BACs) to ≥ 80 mg/dL (NIAAA, 2023). In humans, this typically occurs after 4-5 standard drinks in 2 hours for adult men and women (NIAAA, 2023; SAMSHA, 2021). Evidently, this pattern of excessive and episodic alcohol misuse precipitates a cascade of neurobiological alterations that influence behavior, as will be discussed below.

1.2 Health and Economic Consequences of Excessive Alcohol Consumption

Alcohol Use Disorder (AUD) is a clinical diagnosis characterized by an individual's inability to control or discontinue alcohol use despite experiencing adverse social, occupational, or health effects (NIAAA, 2022). Excessive alcohol consumption results in a multitude of health complications, including fatty liver, alcoholic hepatitis, and cirrhosis (Mayo Clinic, 2022). It also contributes to kidney dysfunction and chronic kidney disease (NIAAA, 2022). Cardiovascular issues such as high blood pressure, heart disease, cardiomyopathy, and stroke are prevalent among heavy drinkers (Pearson, 1996). Additionally, alcohol abuse can lead to pancreatitis, both acute and chronic, and neurological damage, including Wernicke-Korsakoff syndrome, a severe memory disorder caused by thiamine deficiency (NIAAA, 2022). Furthermore, alcohol consumption is a known risk factor for various cancers, including those of the mouth, throat, esophagus, liver, colon, and breast (American Cancer Society, 2020). It weakens the immune system, increasing susceptibility to infections (Mayo Clinic, 2022), and causes digestive problems such as gastritis, ulcers, and intestinal damage (NIAAA, 2022). Moreover, alcohol abuse is linked to mental health disorders, including depression, anxiety, and an increased risk of suicide (NIAAA, 2022). Alcohol withdrawal syndrome (AWS), which occurs when individuals with chronic alcohol use abruptly reduce or stop their alcohol consumption, poses severe short-term effects such as anxiety, tremors, sweating, nausea, seizures, and delirium tremens (DTs), which can be life-threatening (Kosten & O'Connor, 2003; Mayo Clinic, 2022). Long-term effects include persistent cognitive deficits, mood disorders, and an increased risk of relapse (Le Berre et al., 2017; Saitz, 1998).

These significant health complications from excessive alcohol intake result in substantial repercussions in terms of healthcare costs and productivity losses (Bohm et al., 2021; Sacks et al., 2015). In 2010, the Centers for Disease Control and Prevention estimated a $175 billion loss in workplace productivity in the United States alone, highlighting the extensive economic costs of alcohol abuse (CDC, 2018; Sacks et al., 2015). Alcohol abuse also poses significant public health and safety concerns, particularly with respect to road traffic accidents. Francesconi and James (2019) report a correlation between alcohol binge-drinking and an 18.6% increase in road accidents and a 72% increase in traffic-related fatalities, highlighting a severe risk that extends beyond individual users and jeopardizes public safety. Given these statistics, it is evident that the consequences of excessive alcohol consumption extend well beyond the individual user, impacting entire families, communities, and national and global economies. Understanding the biomedical bases for AUD vulnerability is crucial for developing effective prevention and treatment strategies, highlighting the importance of continued research in this field.

1.3 Understanding Adolescent Alcohol Reward Through Psychosocial Influences and Neuropsychiatric Consequences

In the United States, adolescents and young adults are consistently the most likely age group to engage in binge alcohol-drinking (NIAAA, 2017; Patrick & Schulenberg, 2014). Compared to adults, adolescents drink less frequently but consume nearly double the average volume of alcohol per occasion (Chung et al., 2018; SAMSHA, 2021). Additional reports estimate that those aged 12–20 years account for 11% of all alcohol consumed, with over 90% of this consumption occurring through binge drinking (NM-IBIS, 2022). As detailed

below, the high rate of binge-drinking exhibited by adolescents is concerning given that adolescence is a critical period for neurodevelopment (Dorn, 2006; Spear, 2013) and indeed, some evidence exists indicating that alcohol consumption differentially affects the brain in adolescents versus adults (Spear, 2015). Research indicates that the adolescent brain exhibits significantly greater neuroplasticity compared to adults, increasing susceptibility to the detrimental effects of alcohol (Crews et al., 2016; Squeglia et al., 2014). This heightened plasticity can disrupt neural development, leading to long-term behavioral and cognitive vulnerabilities in adulthood, emphasizing age-related differences in alcohol-induced neurotoxicity (Brancato et al., 2021; Tetteh-Quarshie & Risher, 2023).

Although often used synonymously, adolescence and puberty are not interchangeable terms (Spear, 2010). While puberty refers to the phase in which the individual acquires sexual maturity, usually accompanied by neuroendocrine and physiological changes of sexual maturation, adolescence is comprised of the entire transition period from childhood to adulthood (Dorn, 2006; Spear, 2010). This developmental period is characterized by both physical growth and profound psychological and neurodevelopmental changes that contribute to a young person’s sense of identity and independence (Crone & Dahl, 2012). During this period, the brain's frontal regions, crucial for impulse control and judgment, are still developing, and this gap in development renders adolescents particularly vulnerable to partaking in risky behaviors (Novier et al., 2015; Spear & Varlinskaya, 2005), including drug- and alcohol-taking behaviors that can lead to substance use disorders, including AUD (Koob & Volkow, 2009). In essence, the adolescent brain is hardwired for novelty, and these risky behaviors are in part due to an underdeveloped prefrontal cortex, which is responsible

for measuring consequences and controlling impulses (Steinberg, 2005). Increased risk-taking behavior observed in human adolescents extends also to laboratory rodents; adolescent animals approach novel stimuli more quickly, exploring novel situations and stimuli more than their older counterparts, and find novel stimuli to be more rewarding than do adults (Adriani et al., 1998; Douglas et al., 2004). The increased release of dopamine during sensation-seeking activities makes these risks even greater and encourages adolescents to seek out novel, thrilling experiences, including those that carry a high risk, like binge drinking (Koob et al., 2015; Novier et al., 2015; Spear & Varlinskaya, 2005). Adding to this, adolescence is a critical period of neurodevelopment where the brain undergoes fine-tuning that can amplify an adolescent's ability to consume more alcohol by boosting its rewarding properties (Brown & Tapert, 2004; Spear, 2002, 2011; Spear & Varlinskaya, 2005). Evidence from both human and rodent studies has identified various psychological and social factors that contribute to the rewarding effects of alcohol, illustrating a complex and multifaceted interplay between these influences (Anacker & Ryabinin, 2010; Sloboda et al., 2012).

Adolescent-onset alcohol drinking has been associated with heightened vulnerability to mood disorders, including anxiety and depression, in later life (Holmgren & Wills, 2021). In fact, an AUD diagnosis in adolescence is one of the strongest predictors of mood disorders in adulthood (Hanson et al., 2011). This suggests that the emotional processing centers of the brain, like the regions comprising the extended amygdala, are particularly sensitive to the neurotoxic effects of alcohol during adolescence (Brown & Tapert, 2004). Interestingly, while adolescents and young adults are more sensitive to alcohol's rewarding properties, they appear to be more resilient to the immediate negative affective consequences of excessive

alcohol consumption. Research indicates that this resilience may stem from the fact that the neural pathways mediating the aversive properties of alcohol are not fully developed in adolescents (Petit et al., 2013; Spear, 2013, 2014). In humans, adolescents often experience fewer immediate negative emotional effects and hangover symptoms compared to adults, which can reinforce maladaptive drinking behaviors (Spear & Varlinskaya, 2005). This absence of immediate negative consequences may obscure the neurotoxic effects of alcohol, potentially leading to profound and long-lasting consequences for cognitive and emotional functioning. Animal studies support these findings, suggesting that the developing adolescent brain exhibits different responses to alcohol-induced neurophysiological disruptions (Gilpin et al., 2012; Varlinskaya & Spear, 2004). Although adolescents may recover quickly from acute disruptions, this does not protect against the long-term neurodevelopmental perturbations caused by alcohol, which can manifest later in life even in the absence of continued alcohol exposure. As such, my thesis employed a mouse model of binge-drinking to examine how a history of adolescent binge drinking impacts the brain and behavior both in the short and long term.

Understanding adolescent alcohol consumption is a complex challenge that directly reflects the complex nature of human behavior. Decades of research have begun to untangle this complexity, demonstrating that specific genetic and phenotypic vulnerabilities may significantly raise the risk of substance use (Conner et al., 2010; Cozzoli et al., 2012; Miela et al., 2018; Nestler, 2000; Piazza et al., 2000; Szumlinski et al., 2017). For instance, research shows that adolescents with a family history of alcohol abuse are more prone to heavy drinking (Hill et al., 2000). Additionally, individuals with certain personality traits, such as

impulsivity or sensation-seeking behaviors, may be more prone to using alcohol as a coping mechanism for stress or emotional regulation (Magid et al., 2007). Research in both rodent and human studies suggests that various factors contribute to adolescent alcohol misuse, with heightened sensitivity to the rewarding properties of alcohol playing a significant role in its prolonged use and addictive nature (Lee et al., 2015, 2016; Spear, 2011, 2002). Further, an increase in stress and psychological distress have both been frequently reported to contribute to excessive and often problematic alcohol use, with individuals adopting excessive drinking as a self-medication method to alleviate negative emotional states (Gould et al., 2012; Rodriguez et al., 2020). This behavior is consistent with the self-medication hypothesis, which suggests that the use of substances like alcohol offers temporary alleviation from psychological distress (Khantzian, 1997). In support of this hypothesis, studies have demonstrated that the acute anxiolytic and mood-enhancing properties of alcohol render it a preferred substance of choice for those with elevated stress and psychological distress levels (Gould et al., 2012; Rodriguez et al., 2020). Moreover, this coping mechanism, facilitated by excessive alcohol intake, can feed into a maladaptive cycle, predisposing individuals to an elevated risk of developing an AUD (Koob et al., 2015; Le Berre, 2019). Consequently, an adolescent struggling with the pressures of school and social relationships may turn to alcohol to temporarily alleviate feelings of stress and anxiety. I hypothesize herein that, over time, repeated bouts of binge drinking during adolescence are likely to impact the brain's developmental trajectory, ultimately causing emotional and cognitive disturbances in later life.

1.4 Sex Differences in Alcohol's Rewarding Properties and Withdrawal-Induced Neuropsychiatric Consequences

An increasing number of recent studies have highlighted the narrowing gap between the sexes in AUD diagnoses, with recent trends showing an alarming 84% rise in the prevalence of AUD in women over the last decade, in contrast to a 35% increase in men (Grant et al., 2017; Peltier et al., 2019; White, 2020). This significant increase in alcohol use among women is particularly concerning considering that women experience more severe health consequences related to excessive alcohol use than men, including a greater susceptibility to alcohol-related liver damage, increased cancer risks, and a list of cardiovascular complications (Agabio et al., 2017; NIAAA, 2017; Jousilahti et al., 1999).

Interestingly, both sexes differ not only in their consumption patterns but also in their responses to the rewarding properties of alcohol. For example, research suggests that women consume alcohol more frequently than men as a method to cope with emotional distress, whereas men are more likely to drink for social reasons (Nolen-Hoeksema, 2004; Rodriguez et al., 2020). These different consumption patterns are closely linked to the progression of AUD, indicating that women experience more adverse effects at each stage compared to men (Koob, 2009; Verplaetse et al., 2018). For example, the number of alcohol-related emergency department visits between 2006 and 2014 increased by 61.6%, with the annual percentage change in rates of all alcohol-related visits being higher for women than for men (5.3% vs. 4.0%) (White et al., 2018). Additionally, from 1993 to 2010, alcohol-related hospital inpatient diagnoses among adults increased by 90% in women compared to 30% in men, highlighting the disproportionate impact on women (Sacco et al., 2015). Societal changes and

the evolution of gender roles may also play a role in the closing of the gender gap in alcohol drinking as women increasingly participate in traditionally male-dominated social environments where drinking is prevalent (McKetta et al., 2021). Evidence also suggests that the mood-enhancing effects of alcohol, which play a key role in its misuse, are perceived differently by men and women, suggesting biological mechanisms may be underlying these differences in alcohol's interoceptive effects (Fink, 2017; Flores-Bonilla & Richardson, 2020; Rodriguez et al., 2020). Neurobiologically, these variations may relate to sexual dimorphisms of neural circuits related to stress and reward. For instance, rodent models have provided insight into sex-specific alcohol effects in the brain, showing that female rodents develop dependence more quickly and exhibit more severe affective withdrawal symptoms than males, akin to the observations seen in women (Jimenez Chavez et al., 2022; Melón et al., 2013; Szumlinski et al., 2019).

Advancements in understanding the genetic and molecular mechanisms underlying these sex differences have identified specific genes and pathways, particularly those involved in neuroinflammatory responses and neuroplasticity, as exhibiting different expression patterns between males and females following a history of alcohol exposure (Cruz et al., 2023; Flores-Bonilla & Richardson, 2020; Wiren et al., 2006). This differential expression suggests potential biomarkers for AUD that are sex-specific, further entangling the disorder's pathology. Neuroimaging studies have also reported on the different patterns of brain atrophy in women with AUD, specifically in regions implicated in executive functioning and emotional regulation, further highlighting the sex-specific differences in the neural

mechanisms of AUD (Fama et al., 2020; Mann et al., 2005). These differences emphasize the need for sex-specific approaches to understanding and treating AUD.

The disruption of the hypothalamic-pituitary-adrenal (HPA) axis by chronic alcohol consumption further illustrates the complexity of these sex differences. Females with a history of excessive alcohol intake are known to exhibit heightened HPA axis responses to stress compared to males, potentially contributing to differences in drinking behavior and relapse rates between sexes (Peltier et al., 2019). Furthermore, stress response systems, including the HPA axis, exhibit sexual dimorphism and are influenced by alcohol consumption. Implicating these mechanisms in the challenges related to stress responses and mood dysregulation commonly observed in addiction pathology (Jimenez Chavez et al., 2020; Knezevic et al., 2023), studies show that alcohol-induced dysregulation of the HPA axis is more pronounced in females and contributes to increased severity of withdrawal symptoms and increased relapse rates in women (Silva & Madeira, 2012; Silva et al., 2009). This heightened stress response in women may exacerbate inflammatory processes in the brain, leading to sex-specific neuroinflammatory profiles. Chronic alcohol intake is also associated with sex-specific differences in inflammatory responses in the brain, manifesting different pathophysiological profiles. Preclinical research indicates that females with a history of heavy drinking exhibit elevated levels of pro-inflammatory cytokines, such as interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6), compared to their male counterparts (Pascual et al., 2016). This heightened inflammatory response observed in females may exacerbate neurodegenerative mechanisms and accelerate cognitive decay in women versus men, emphasizing the sex-specific divergence in the

neuropathological consequences of excessive alcohol consumption (Cruz et al., 2023; Flores-Bonilla & Richardson, 2020).

Moreover, differences in neurotransmitter systems, particularly those involving glutamate, play a pivotal role in AUD. Research into glutamate neurotransmission has shown that chronic alcohol use elicits neuroadaptive changes in glutamatergic signaling pathways, with evidence indicating that these changes are potentiated by interactions with sex hormones like estrogen and progesterone, resulting in more adverse changes in females (Flores-Bonilla & Richardson, 2020; Kovacs & Messingham, 2002). These changes are associated with an increased vulnerability to alcohol's neurotoxic effects, which can intensify over time and are especially detrimental during neurodevelopment. This facilitates the onset of withdrawal symptoms and accelerates the progression to addiction (Becker, 2016; Quigley et al., 2021). Additionally, alterations in glutamate receptor trafficking and post-translational modifications have been shown to be significant contributing factors to the heightened vulnerabilities seen in females (Fabian et al., 2023). Glutamate receptor subtypes, such as N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, have shown sex-dependent alterations in functioning due to alcohol exposure, influencing neural plasticity, learning, and addiction-related behaviors (Szumlinski et al., 2023a; Finn et al., 2018).

1.5 Studying Binge-Drinking and its Biopsychological Consequences in Rodent Models

Animal behavioral models, particularly rodents, are used to simulate biobehavioral responses that are seen in humans (Jaggi et al., 2011). These models are important for several

reasons. Rodents share significant genetic, biological, and behavioral similarities with humans, making them invaluable for studying complex human behaviors and psychiatric conditions (Sousa et al., 2006). One of the primary benefits of rodent models is their ability to enable controlled manipulation of variables and in-depth longitudinal studies, which are often impractical in human research (Cavigelli, 2005). Rodent models facilitate the study of gene-environment interactions by allowing researchers to manipulate genetic backgrounds and environmental conditions to observe resultant behaviors and neurological changes (Crawley, 1999; Tarantino et al., 2011). This is particularly valuable for understanding the development of psychiatric disorders and the identification of potential therapeutic targets (Cryan & Mombereau, 2004). Rodent models are also instrumental in the preclinical testing of pharmacological treatments for psychiatric disorders, providing critical data on efficacy and safety before human trials (Cryan & Mombereau, 2004; Ramaker & Dulawa, 2017). Ultimately, these models have significantly contributed to understanding the biobehavioral mechanisms underlying substance use disorders, including withdrawal symptoms and affective disturbances including anxiety- and depression-like symptoms (Becker & Ron, 2014).

Behavior-contingent or voluntary alcohol self-administration in rodent models is advantageous because it more closely mimics human patterns of alcohol consumption. This method allows researchers to study the motivational aspects of alcohol intake and its behavioral consequences under conditions that are more representative of voluntary human drinking (Becker & Ron, 2014; Rhodes et al., 2005). Behavior-contingent self-administration models also provide valuable insights into the neurochemical and behavioral changes

associated with voluntary alcohol intake, which can differ significantly from those observed with noncontingent administration methods (Kippin et al., 2006; Lecca et al., 2007; Tabakoff & Hoffman, 2000). In this regard, the Drinking-in-the-Dark (DID) paradigm is an established and effective model for studying voluntary binge-like alcohol consumption in rodents. As detailed by Rhodes et al. (2005), this model involves housing rodents in a 12-hour light, 12-hour dark cycle. Ethanol access is provided 3 hours into the dark cycle, which aligns with the rodents' peak activity period, promoting binge drinking behavior. This reliably induces high blood alcohol concentrations comparable to those seen in human binge drinking episodes. In the DID paradigm, animals are individually housed during drinking sessions and receive limited access (~ 2h) to ethanol over several days. The ethanol is delivered via sipper tubes or bottles using a multi-bottle choice approach with varying concentrations of unsweetened ethanol ranging from 10% to 40%. This setup allows researchers to investigate the acute and chronic effects of binge drinking on both behavior and neurobiology, providing valuable insights into the mechanisms underlying AUDs and their consequences (Rhodes et al., 2005; Thiele & Navarro, 2014). For these reasons, my dissertation research employs DID procedures to examine both the short- and long-term effects of binge drinking during adolescence on the brain and behavior.

Various rodent models exist that enable the study of the biobehavioral responses during alcohol withdrawal and have been used to provide insight into neuropsychiatric disorders comorbid with AUD, including anxiety-like responses aimed at modeling human pathological anxiety (Becker, 2000). There are various ways of inducing an anxiety-like response in rodents. Some of the most common methods involve subjecting rodents to an

aversive stimulus or event or by presenting signs of a perceived aversive stimulus/event (Bailey & Crawley, 2009; Lezak et al., 2017). One such method is the conditioned place aversion (CPA) paradigm, which assesses the aversive effects of a stimulus. In the context of alcohol withdrawal, rodents are conditioned to associate a specific environment with the aversive effects of withdrawal. During the conditioning phase, rodents are exposed to one environment where they experience withdrawal symptoms and another neutral environment. Following conditioning, the preference for each environment is assessed. The avoidance of the withdrawal-paired environment serves as a measure of the aversive properties of withdrawal (Cunningham & Hendersen, 2000; Prus et al., 2009).

Other tests integrate an approach-avoidance conflict that leverages rodents' natural avoidance of brightly illuminated (protected) areas and instinctive behavior to explore unfamiliar environments (Bailey & Crawley, 2009). One such test is the Elevated Plus Maze (EPM), a widely used assay for measuring anxiety-like behavior in rodents (Kraeuter et al., 2019). The EPM consists of two open arms and two enclosed arms arranged in a plus shape, elevated above the floor. During the test, rodents are placed in the center of the maze, and their behavior is observed. Typically, rodents avoid the open arms due to their aversion to open, brightly lit spaces and prefer the enclosed arms. Increased time spent in the open arms is interpreted as reduced anxiety-like behavior, while reduced time indicates heightened anxiety-like behavior, which can be particularly pronounced during alcohol withdrawal (Kang et al., 2017; Pellow et al., 1985). Another common test is the Open Field Test (OFT), which involves placing a rodent in a large, brightly lit arena. The rodent's activity is monitored to assess its exploratory behavior and anxiety levels. Normally, rodents spend

more time near the walls (thigmotaxis) and avoid the center of the open field due to their fear of open spaces. Increased center exploration is indicative of reduced anxiety, whereas decreased exploration suggests heightened anxiety, a response often observed during alcohol withdrawal (Overstreet et al., 2004; Prut & Belzung, 2003). The light-dark box shuttle test was first introduced by Crawley and Goodwin in 1980 as a simple animal behavior model to test the anxiolytic effects of benzodiazepines (Crawley & Goodwin, 1980). Based on the observation that rodents, when given a choice, tend to avoid bright and open spaces—presumably due to perceived exposure to predators—there is a corresponding decrease in exploratory behavior (Crawley & Goodwin, 1980; Valzelli, 1969). This assay features an apparatus with two chambers: a covered, dark side (protected side) and an uncovered, brightly illuminated side (unprotected side), with a passageway between both chambers that allows the animal to easily access either side. Shorter latencies to enter the light side, more time spent in the light side, and an increased number of entries to the light side are interpreted as indicative of less anxiety-like behavior or as having an anxiolytic effect (Lezak et al., 2017). As expected, treatment with various anxiolytic drugs reduces avoidance behavior and increases exploratory behavior in the light side (Bourin, 2001; Crawley & Goodwin, 1980).

Neophobia manifests as an innate or learned fear response to novel stimuli or environments, which is a common trait of both humans and laboratory rodents (Mettke-Hofmann, 2022). Various assays are used to study neophobia-related behaviors in rodent models, which include measurements of defensive burying. In rodents, defensive burying occurs when a rodent encounters a potential threat; their innate response is to bury the object

as a coping mechanism to reduce its perceived threat (De Boer & Koolhaas, 2003). This anxiety-like behavior represents an active defensive response to perceived dangers or stressors in an environment. One assay that measures this behavior is the Shock Probe Defensive Burying Test. In this test, a rodent is placed in an environment with a probe that delivers mild electric shocks. The rodent's response to the shock is to bury the probe using available bedding material. The amount of bedding used, and the time spent burying are recorded. This test is particularly relevant in the context of alcohol withdrawal, as it can reveal heightened anxiety-like responses (Treit et al., 1981). Rodents undergoing withdrawal typically exhibit increased burying behavior, indicating elevated anxiety (De Boer & Koolhaas, 2003; Fucich & Morilak, 2018). Another related assay is the Novel Object Reactivity Test, which evaluates a rodent's response to a new, unfamiliar object placed in its environment. This test is useful for assessing exploratory behavior and anxiety levels. Generally, compared to control animals, diminished interaction with and exploration of a novel object is considered indicative of heightened anxiety (Hoplight et al., 2005; Ropartz & Misslin, 1981). During alcohol withdrawal, rodents often display increased neophobia, spending less time interacting with the novel object and more time avoiding it. The latency to approach and the time spent interacting with the novel object are key measures. Heightened neophobic responses during withdrawal are indicative of increased anxiety-like behavior (Rasmussen et al., 2001). Additionally, in the marble burying assay, levels of anxiety-like responses are measured through observations of burying activity. Here, burying more marbles can be interpreted as a direct manifestation of a rodent's attempt to manage anxiety by controlling its environment (Himanshu et al., 2020). In other words, the higher the anxiety-like behavior, the shorter the latency to bury marbles, and the more time and number

of marbles buried. A relevant debate in the literature suggests that marble-burying behavior is more indicative of a “compulsion-like” behavior than an anxiety-like response (Gyertyán, 1995; Londei et al., 1998). However, numerous studies have shown this assay to be useful in measuring novelty-induced anxiety, and pharmacological evidence links an inhibition of marble burying to the administration of anxiolytic agents including selective serotonin-reuptake inhibitors (SSRIs) and tricyclic antidepressants, among others (Archer et al., 1987; Broekkamp et al., 1986; Ichimaru et al., 1995; Millan et al., 2001).

The validity of rodent models for assessing depression-like behaviors is a subject of ongoing debate within the scientific community. Identifying measurable signs of depression in rodents presents significant challenges, and there is a notable lack of fully validated models in the existing literature (Castagné et al., 2010; Porsolt et al., 1977). Despite these limitations, several animal models have been developed to measure depressive-like behaviors, which are crucial indicators of the negative emotional state experienced during alcohol withdrawal in rodents. One such model is the Tail Suspension Test (TST), which is widely used to assess behavioral despair in rodents (Can et al., 2012). In this test, a rodent is suspended by its tail, and its movements are recorded. Typically, rodents will initially struggle to escape but eventually exhibit immobility, which is interpreted as behavioral despair. During alcohol withdrawal, increased immobility time is observed, indicating heightened depressive-like behavior. This model is particularly relevant for studying the neurobiological mechanisms of depression during withdrawal (Can et al., 2012; Steru et al., 1985; Tonetto et al., 2023). Another common model used to measure anhedonia, a core symptom of depression, is the Sucrose Preference Test. This test assesses a rodent's

preference for a sweet sucrose solution over plain water. A decreased preference for the sucrose solution is interpreted as anhedonia (Papp et al., 1991; Serchov et al., 2016). During alcohol withdrawal, rodents often exhibit reduced sucrose preference, indicating a loss of interest in or pleasure in normally rewarding activities. This model is essential for understanding the affective components of depression associated with withdrawal (Der-Avakian & Markou, 2012; Katz, 1982).

One of the most widely utilized behavioral assays in the context of modeling depressive-like behavior is the Porsolt Forced Swim Test (FST). The FST evaluates "behavioral despair," which serves as a proxy for a depression-like state in rodents (Porsolt et al., 1977). Although the term "behavioral despair" is used in the absence of fully validated depression models, it provides a valuable metric for assessing depressive-like behaviors in rodent studies. In humans, depression often manifests as feelings of hopelessness and pessimism (Nekanda-Trepka et al., 1983), which is presumed to be modeled in the FST. In this assay, rodents are placed in a water-filled cylindrical container, creating an inescapable environment. Initially, the animals typically exhibit panic-like swimming behavior in an attempt to escape. However, as the animals learn the inescapable nature of the swim tank, they shift from this active coping strategy to passive floating, which is interpreted as a sign of despair or resignation that is indicative of a depressive-like state (Castagné et al., 2010; Porsolt et al., 1977). Using the FST, the measurement of passive and active coping strategies can also be studied during withdrawal periods. Active coping strategies are characterized by prolonged escape attempts, whereas passive coping strategies are marked by increased periods of immobility and/or a quicker latency to the first immobile state (De Kloet &

Molendijk, 2016; Warden et al., 2012). This distinction between active and passive coping behaviors is profoundly relevant to human behavior (Suls & Fletcher, 1985). Active coping involves behavioral and cognitive efforts to deal with problems and mitigate their impacts directly (Carver et al., 1989). In contrast, avoidant/passive coping refers to strategies aimed at avoiding direct confrontation with problems, often resulting in behaviors like excessive drinking to alleviate emotional stress (Blalock & Joiner, 2000; Fromme & Rivet, 1994). As suggested by several studies, in both humans and rodents, passive coping strategies are often adopted when subjects perceive their situation as unchangeable, warranting acceptance of their circumstances (Blalock & Joiner, 2000; Warden et al., 2012). Supporting the predictive validity of floating behavior in the FST, pretreatment with a number of different antidepressant drugs (including ketamine) reduces the amount of passive floating exhibited by the animal (Fitzgerald et al., 2019; Slattery & Cryan, 2012). Furthermore, the FST offers avenues to study anxiety-like behaviors, especially in the context of alcohol withdrawal (Lee et al., 2015, 2016, 2017b; Szumlinski et al., 2018). Lee et al. (2017b) provided evidence that alcohol-withdrawn mice show a decrease in passive floating, which is associated with increased anxiety-like behavior. However, when treated with anxiolytic agents, such as buspirone and MTEP, mice displayed a significant increase in immobility. This shift from active swimming to passive floating underscores the predictive validity of the FST as an effective model for assessing anxiety.

Despite extensive research on the behavioral consequences of alcohol withdrawal and its age-dependent variations, there remains a significant gap in the literature regarding the investigation of both age and sex differences in these effects. This represents an important,

yet underexplored, area of research. To address this gap in the literature, my thesis will specifically focus on the anxiety responses that are dependent on age (adolescent vs. adult) following a history of binge-drinking, with a particular emphasis on the role of sex differences (male vs. female). Building on the foundational work conducted by the Szumlinski laboratory (Lee et al., 2015, 2016, 2017a,b, 2018a,b,c), which has demonstrated that the Forced Swim Test (FST), Marble Burying Test (MBT), and Light-Dark Box (LDB) are the most consistently sensitive assays for evaluating withdrawal-induced anxiety-like behaviors, my research will employ these methodologies to assess anxiety-like responses.

1.6 Neurobiology of Alcohol Use Disorder and Withdrawal-induced Negative Affect

Fundamentally, alcohol's effect on the brain involves its ability to modulate different neurotransmitter systems. Alcohol primarily enhances gamma-aminobutyric acid (GABA) inhibitory signaling and suppresses the activity of the excitatory neurotransmitter glutamate (Valenzuela, 1997). This dual action is key to understanding the sedative and anxiolytic effects of acute alcohol (Becker & Mulholland, 2014). Alcohol acts as a co-agonist of the GABA A receptor and increases chloride ion influx, hyperpolarizing neurons and leading to decreased neuronal excitability (Kumar et al., 2009; Lobo & Harris, 2008). In addition to its direct action on GABA A receptors, another key molecular target implicated in alcohol's effects on the brain are NMDA receptors. NMDA receptors are ionotropic glutamate receptors composed of multiple subunits that form heterotetrameric complexes that play a key role in synaptic plasticity, learning, and memory (Cull-Candy et al., 2001; Hansen et al., 2017). The GluN1 subunit is important for the functional assembly of NMDA receptors, while the GluN2 subunits (A-D) confer distinct biophysical and pharmacological properties.

Specifically, the GluN2B subunit is particularly sensitive to alcohol (Woodward, 2000) and, thus, has been extensively studied for its role in alcohol-related behaviors. Alcohol inhibits NMDA receptors in a non-competitive manner, reducing excitatory neurotransmission (Woodward, 2000). Additionally, alcohol is reported to inhibit metabotropic glutamate receptors mGlu1 and mGlu5 (Mitsukawa et al., 2005), which are Gαq-coupled receptors also highly implicated in neuroprotection as well as synaptic plasticity associated with learning and memory. These actions collectively contribute to the overall depressant effects of acute alcohol on the central nervous system.

With chronic alcohol use, neurochemical alterations occur that promote homeostatic deregulation, resulting in an allostatic shift in brain activity (Koob & Le Moal, 2001). The onset of psychiatric symptoms indicative of, and comorbid with, an AUD has been linked to compensatory changes related to an overactive glutamate system that can persist in the absence of alcohol (Bell et al., 2016; Bergink et al., 2004). This glutamate overactivity is prominent within the extended amygdala, which consists of the central nucleus of the amygdala (CeA), the bed nucleus of the stria terminalis (BNST), and the nucleus accumbens shell subregion (AcbSh), which gate both the rewarding properties of alcohol and the regulation of motivation and emotion (Koob, 2003). For example, chronic alcohol exposure can induce robust increases in the activity of extended amygdala structures, including the CeA, which is theorized to contribute to heightened anxiety and emotional dysregulation (Koob, 2003; Roberto et al., 2012). Similarly, the BNST plays a crucial role in modulating negative affect, such as anxiety and depression, during alcohol withdrawal and abstinence by integrating sensory, emotional, and motivational information and altering inhibitory and

excitatory neurotransmission, highlighting its importance in alcohol-induced emotional disturbances and relapse (Wills, 2019). The nucleus accumbens (NAc) also plays a vital role in this circuitry, with increased activity in the NAc shell during withdrawal contributing to the negative affect associated with chronic alcohol use (Purohit et al., 2018). Of relevance to this thesis, repeated bouts of binge drinking under Scheduled High Alcohol Consumption (SHAC) procedures induce a sensitization of alcohol-induced glutamate release within the nucleus accumbens of male mice (Szumlinski et al., 2007), while repeated bouts of binge drinking under both SHAC (Cozzoli et al., 2009) and DID procedures (Campbell et al., 2019; Cozzoli et al., 2012, 2014a,b, 2016) increase the expression of NMDA, mGlu1, and mGlu5 glutamate receptors, as well as their scaffolding protein Homer2a/b, within all components of the extended amygdala in male mice. Moreover, these glutamate-related protein changes are critical for the manifestation of binge-drinking under both paradigms, as indicated by the results of neuropharmacological studies (Campbell et al., 2019; Cozzoli et al., 2009, 2012, 2014a,b, 2016; Lum et al., 2014). Thus, a chronic history of binge-drinking is sufficient to augment both pre- and postsynaptic aspects of glutamate transmission within the extended amygdala that drive subsequent excessive alcohol consumption.

Alcohol-induced glutamate hyperexcitability within the extended amygdala is also considered key for the development of withdrawal symptoms, including anxiety, irritability, and seizures (Becker & Mulholland, 2014; Bergink et al., 2004; Hoffman, 1995). These symptoms often drive individuals to continue alcohol use to delay or reverse the onset of withdrawal-associated discomfort (Rao et al., 2015; Roberto et al., 2012). Further, repeated bouts of alcohol consumption and withdrawal can result in long-lasting neurochemical

alterations that promote an overactive glutamate system and persistent changes in brain structure and function (Koob & Le Moal, 2001), both within the extended amygdala (Koob, 2014; Lee et al., 2015) and in key brain regions gating executive function, learning, and memory such as the prefrontal cortex (Crews & Boettiger, 2009; Medina et al., 2008), entorhinal cortex (Ibáñez et al., 1995; Crews et al., 2000), and hippocampus (De Bellis et al., 2000; Nagel et al., 2005). Evidence shows that a history of excessive alcohol intake through intragastric administration in rats induced significant increases in mGlu1 receptor activity, which is implicated in the manifestation of an increased negative affective state during alcohol withdrawal (Sánchez-Marín et al., 2022). Moreover, mGlu1 antagonists have been shown to reduce the rewarding effects of alcohol and decrease withdrawal-induced seizures, suggesting a potential therapeutic for treating AUD (Kotlinska et al., 2011; Luessen & Conn, 2022). Further, binge-drinking upregulates mGlu5 receptor expression within the AcbSh and CeA during both early and later withdrawal (Cozzoli et al., 2009, 2012; Lee et al., 2016, 2017b), and inhibition of mGlu5 receptors within the AcbSh attenuates withdrawal-induced anxiety (Lee et al., 2018c). The mGlu1 and mGlu5 subtypes of glutamate receptors are scaffolded by members of the Homer family of proteins, which regulate their synaptic localization and signaling capacity (Szumlinski et al., 2008a). Repeated alcohol exposure, including binge-drinking, increases the expression specifically of the Homer2a/b isoform within the AcbSh, BNST, and CeA (Campbell et al., 2019; Cozzoli et al., 2009, 2012, 2014a; Goulding et al., 2011; Szumlinski et al., 2008b), while reduced Homer2a/b expression is associated with indices of negative affect during both early and late withdrawal (Lee et al., 2016, 2017a). Arguing an active role for Homer2 within the CeA in withdrawal-induced

negative affect, virus-mediated upregulation of Homer2b in alcohol-naïve mice elicits an anxiogenic state and promotes binge drinking (Lee et al., 2018a).

Preclinical studies have also demonstrated that repeated binge-like alcohol exposure alters GluN1 expression and function within brain regions associated with emotional regulation that contribute to the heightened anxiety observed during alcohol withdrawal (Fadda & Rossetti, 1998). Studies using rodent models of binge drinking have revealed that chronic alcohol exposure leads to an increase in GluN2B receptor expression in the prefrontal cortex and amygdala (Cozzoli et al., 2009, 2012; Kash et al., 2009; Lee et al., 2016, 2017a; Roberto et al., 2006) that is associated with heightened anxiety and depressive-like behaviors during withdrawal (Lee et al., 2016, 2017a) as well as excitotoxicity and stress responses (Boyce-Rustay & Holmes, 2006). Thus, while elevated corticotropin-releasing factor (CRF) has been highly implicated in priming the brain for hyperkatifeia (i.e., a state of heightened negative emotionality during withdrawal) (Koob, 2021), abundant evidence supports a necessary and active role for glutamate hyperactivity in contributing to the complex neurobiology of withdrawal.

1.6.1 Role of Age in Withdrawal-induced Negative Affect.

As discussed above, early alcohol exposure has been shown to disrupt normal brain development, leading to structural changes in key brain regions involved in emotional regulation and behavior that increase susceptibility to anxiety and depressive-like behaviors in later life (Lees et al., 2019; Pandey et al., 2015). Adolescents often exhibit resilience to the immediate negative affective effects of alcohol withdrawal, showing fewer signs of anxiety

and depression compared to adults during the acute withdrawal phase (Wills, 2009). This resilience is in part attributed to the developmental differences in neurobiological systems, such as the HPA axis and neurotransmitter systems, which are still maturing during adolescence (Spear, 2015). Studies have shown that the adolescent brain is more adaptable and can recover more quickly from the neurochemical disruptions caused by alcohol (Spear, 2000a). This resilience is reflected in milder behavioral and cognitive impairments observed during earlier withdrawal phases in adolescent animals compared to their adult counterparts (Crews et al., 2007; Lee et al., 2016, 2017a, 2018b; Silveri & Spear, 2002). However, this temporary resilience does not imply long-term protection, as this resilience does not persist into adulthood. While adolescents may appear resilient to the immediate affective disturbances caused by alcohol withdrawal, the enduring neurobiological alterations they experience lay the foundation for significant mental health challenges as they transition into adulthood. Indeed, preclinical studies indicate that the neurobiological changes induced by alcohol during adolescence can result in an increased vulnerability to anxiety and mood disorders later in life (Sakharkar et al., 2016). For instance, alterations in the GABAergic and glutamatergic systems can persist into adulthood, leading to heightened stress reactivity and anxiety disorders (Ehlers & Criado, 2010; Gilpin et al., 2015). Binge drinking studies from the Szumlinski laboratory have corroborated the observation that adolescent male mice are resilient to the negative affective state during early alcohol withdrawal, but also revealed that the adverse effects of an adolescent history of binge-drinking on affect do not manifest until protracted withdrawal when the mice are young adults (i.e., the negative affective state incubates during protracted withdrawal) (Lee et al., 2016, 2017a, 2018a,b). In support of a potential sex difference in this incubation, a study of female mice indicated that a negative

affective state manifests during both adolescence and young adulthood in mice with a history of adolescent-onset binge-drinking (Szumlinski et al., 2019).

Excessive alcohol consumption during adolescence is known to disrupt the balance of neurotransmitter systems in the long-term, which might drive disordered behavior in later life. For instance, early alcohol exposure leads to long-term changes in dopamine receptor expression and function, which has been argued to increase the risk of developing substance use disorders later in life (Hauser et al., 2021; Spear, 2015) and predispose individuals to depression and anxiety in adulthood, long after the cessation of alcohol use (Pandey et al., 2015). Moreover, glutamatergic and GABAergic systems, essential for synaptic plasticity and overall brain excitability, are also affected, resulting in persistent changes that impair cognitive function (Abrahao et al., 2017; Maldonado-Devincci et al., 2010) and sensitize the activation of stress-related neurocircuits that exacerbate psychiatric symptoms (Gilpin & Weiner, 2017; Sarawagi et al., 2021). An increase in overall brain excitability also appears to drive the latent effects of adolescent alcohol exposure on emotional reactivity, as evidenced by the results of immunoblotting studies indicating, at least in male mice, that a history of adolescent binge-drinking does not affect glutamate receptor-related protein expression within the AcbSh or CeA during early withdrawal when the adolescent mice are exhibiting low levels of withdrawal-induced negative affect but induces latent changes in protein expression within these regions in later life at a time coinciding with high levels of negative affect (Lee et al., 2016, 2017a). These latter results argue that the age of drinking onset is a critical factor influencing the latency of glutamate anomalies that drive the long-term affective consequences of binge-drinking.

1.6.2 Role of Sex in Withdrawal-Induced Negative Affect.

Given the rapidly decreasing gender gap in alcohol addiction, research into sex differences in response to alcohol and subsequent withdrawal is an emerging and complex topic. Studies suggest that men and women may experience varying levels of glutamate hyperexcitability and anxiety during alcohol withdrawal that result in more severe affective dysfunctions for women (Kasten et al., 2020; Peltier et al., 2019; Verplaetse et al., 2018). Hormonal differences, mainly estrogen and progesterone, are known to modulate the glutamatergic system and may account for variations in withdrawal symptoms between the sexes (Goyette et al., 2023). Estrogens and their receptors are known to provide neuroprotective effects by counteracting the glutamatergic hyperactivity associated with withdrawal-induced anxiety (Lan et al., 2014; Zhao & Brinton, 2007). During the hyperexcitable state precipitated by alcohol withdrawal, estrogen's modulation of these receptors is theorized to dampen the neural response to excess glutamate (Miller et al., 2020), thus potentially alleviating some of the adverse effects on mood and behavior. Further, progesterone is reported to have anxiolytic properties and to modulate stress and anxiety responses (Stefaniak et al., 2023). Interestingly, recent evidence suggests that progesterone may exacerbate nicotine withdrawal and withdrawal-induced negative affect in men while showing no such influence in women (Novick et al., 2022). In women, changes in progesterone during the menstrual cycle could contribute to variations in withdrawal symptoms (Hayaki et al., 2020). During the late luteal phase of the menstrual cycle, females may be more vulnerable to the anxiogenic effects of alcohol withdrawal (Handy et al., 2022; Hayaki et al., 2020), which can be then made worse by glutamate hyperexcitability.

Testosterone may also influence the regulation of glutamate receptors and neural homeostasis (Goyette et al., 2023). This hormonal difference could result in a distinct pattern of withdrawal symptoms, including the propensity for increased aggression and a different profile of anxiety compared to their female counterparts (Assari et al., 2014; Erol et al., 2019). Sex-specific neurobiological pathways also influence how males and females differ in their stress response during withdrawal. The interaction between stress hormones and the glutamatergic system may be a result of different behavioral outcomes for men and women during withdrawal (Giacometti & Barker, 2020; Flores-Bonilla & Richardson, 2020). For example, males might show a propensity toward aggressive behaviors, while females may exhibit higher levels of anxiety and depressive-like behaviors during withdrawal (Becker et al., 2017; Sontate et al., 2021). Additionally, sex differences in pharmacokinetics, which is how the body absorbs, distributes, metabolizes, and eliminates alcohol, have been shown to contribute to the varying experiences of withdrawal (Agabio et al., 2017). Generally, females often have lower levels of alcohol dehydrogenase, the enzyme involved in metabolizing alcohol (Soldin & Mattison, 2009), and a higher body fat percentage and lower water content than males (Young & Tensuan, 1963), which leads to higher BACs, a slower metabolism of alcohol, and a longer duration of its effects on the brain, thus impacting the glutamate system. This prolonged exposure sensitizes the glutamate system differently, potentially leading to more pronounced withdrawal symptoms (Sharrett-Field et al., 2013; Wilhelm et al., 2016) compared to males.

In rodent models, sex differences are evident in alcohol consumption patterns, with females typically having higher alcohol intakes than males (Juárez & Barrios de Tomasi, 1999; Jury et al., 2017; Melón et al., 2013; Szumlinski et al., 2019). This difference in consumption can exacerbate the severity of withdrawal symptoms in females. For example, Varlinskaya and Spear (2015) found that female rodents consumed more alcohol and exhibited more severe withdrawal symptoms compared to males. Evidence from rodent studies also indicates significant sex differences in the stress response system and its interaction with glutamatergic pathways, which are critical in the context of alcohol withdrawal (Logrip et al., 2018; Sharrett-Field et al., 2013). The HPA axis, responsible for regulating stress responses, exhibits sex-specific responses to glutamate hyperexcitability. This hyperexcitability influences the severity and manifestation of withdrawal-induced anxiety (Becker & Koob, 2016). The HPA axis and the glutamate system are interconnected, with disruptions in glutamate neurotransmission affecting the release of stress hormones. During withdrawal, elevated glutamate levels trigger an overactive HPA axis, leading to a more pronounced stress response. Sex differences are evident in both acute and protracted withdrawal phases. During acute withdrawal, characterized by the immediate symptoms following cessation of alcohol intake, female rodents exhibit greater anxiety-like behavior compared to males in several behavioral assays. (Li et al., 2019; Szumlinski et al., 2019). This acute phase is marked by severe fluctuations in neurotransmitter systems, including increased glutamatergic activity and reduced GABAergic function, which are more pronounced in females (Valdez et al., 2002; Sharrett-Field et al., 2013). Females also show more severe affective disturbances during protracted withdrawal (Holleran & Winder, 2017; Li et al., 2019; Szumlinski et al., 2019). Studies have shown that female rodents continue to

exhibit elevated corticosterone levels and anxiety-like behaviors after cessation of alcohol consumption and, upon re-access to alcohol, will consume more than males (Li et al., 2019), indicating prolonged dysregulation of the HPA axis and sustained neurochemical imbalances (Logrip et al., 2018). The modulating effects of sex hormones like estrogen play a crucial role in these differences. Estrogen has been shown to sensitize the HPA axis to stress, leading to heightened anxiety during withdrawal. For instance, ovariectomized female rats supplemented with estradiol exhibit increased anxiety-like behavior in the EPM compared to those without hormone replacement, highlighting the role of estrogen in modulating stress responses during withdrawal (da Silva et al., 2014; Marcondes et al., 2001).

While there are clear sex differences in the propensity to consume alcohol and in the interactions between the stress and glutamate systems that might drive sex differences in affective symptoms during both acute and protracted withdrawal, at the outset of this thesis, there had been no direct examination of sex differences in the affective consequences of a prior history of binge-drinking as studies had been conducted exclusively in males (e.g., Lee et al., 2015, 2016, 2017a) or in females (Szumlinski et al., 2019). Based on the evidence presented in this subsection, my dissertation project sought to characterize the interactions between the age of binge drinking onset and sex in the manifestation of negative affect. but during early and later withdrawal.

1.7 Specific Aims of Dissertation Research

Age of binge-drinking onset and biological sex are key factors that modulate the effects of alcohol binge drinking on behavior and brain function. This dissertation presents

research designed to explore these interactions in detail. The research conducted and presented in this dissertation was designed to address the following specific aims: (1) to replicate previous findings of age-related effects of binge drinking on negative affect observed in male mice by Lee et al. and extend those findings to female mice; and (2) to investigate sex differences in the expression of negative affect during early and protracted withdrawal periods. (3) Determine how sex-specific chemosensory stimuli (odors from male and female mice) affect marble-burying behavior in adolescent and adult, male and female, sex-naïve B6 mice. (4) Revisit the interaction between the age of binge-drinking onset and biological sex in the expression of alcohol withdrawal-induced negative affect, testing males separately from females during early and protracted alcohol withdrawal; and (5) examine the interaction between age of binge-drinking onset and biological sex in cognitive function expressed during early and protracted alcohol withdrawal. (6) Investigate how a history of binge drinking during adolescence interacts with biological sex to accelerate the progression of affective and cognitive dysfunctions during the normal aging process; and (7) characterize classic molecular biomarkers of neurodegeneration in the entorhinal cortex, hippocampus, prefrontal cortex, and amygdala, and provide correlates to the behavioral findings from Aim 6. Collectively, these studies seek to fill existing gaps in the literature by elucidating the intricate interactions between age of drinking onset, biological sex, and the neurobehavioral and molecular consequences of alcohol binge drinking.

Chapter 2:

Incubation of negative affect during protracted alcohol withdrawal is age-, but not sex selective

2.1 Introduction

Binge-drinking is the most common form of alcohol abuse amongst adolescents. The National Institute of Alcohol Abuse and Alcoholism (NIAAA) cites that 90% of all underage drinkers within the United States have engaged in binge-drinking behaviors (NIAAA, 2004), with an estimated 1 million adolescents engaging in frequent binge-drinking episodes (NIAAA, 2018). Binge-drinking is a pattern of high alcohol consumption that results in blood alcohol concentrations (BAC) ≥ 0.08 g/dL, in an approximately two-hour period (NIAAA, 2004). For humans, this usually occurs after 4 drinks for adult women and 5 drinks for adult men (NIAAA, 2004). The prevalence of binge-drinking in adolescents is concerning as adolescence is a critical period of brain development that occurs in between the ages of 12–17 in humans and approximately postnatal days (PND) 28–50 in laboratory rodents. During this period, the brain undergoes robust structural and functional changes, including alterations in neuronal connectivity and synaptic plasticity (Guerri & Pascual, 2010). With these neuroadaptations come changes in behavior, including increased risk-taking, impulsivity, and vulnerability to stressors (Spear, 2002; Kelley et al., 2004; Steinberg et al., 2008). Coupled with environmental and social influences, these adolescent-related behavioral phenotypes have been theorized to increase drug abuse propensity, including excessive alcohol-drinking (Novier et al., 2015). This increased propensity to consume alcohol is augmented by the fact that adolescents tend to be significantly less sensitive to alcohol's negative reinforcing properties than adults, including "hang-over" and increased negative affect during early alcohol withdrawal (Varlinskaya & Spear, 2004; Steinberg et al., 2008; Lee et al., 2016).

Excessive alcohol experience during adolescence impinges upon neurodevelopment, particularly that within the mesocorticolimbic system regulating motivation, emotion, learning, and memory, as well as volitional control over behavior (Squeglia et al., 2014; Lees et al., 2019). Consequently, excessive alcohol consumption during adolescence is associated with decreased academic performance (Smith et al., 2014; Conegundes et al., 2020), increased criminal activity (Dukes & Lorch, 1989), increased vulnerability to develop affective and substance use disorders in later life, including Alcohol Use Disorder (AUD) (Crews et al., 2000; Brown & Tapert, 2004; Blakemore, 2008; Steinberg et al., 2008; Ali et al., 2010). Additionally, individuals with an early age of drinking-onset suffer from alterations in hypothalamo–pituitary–adrenal (HPA) axis function, which also contributes to the manifestation of affective disorders in humans (Aoki et al., 2010; Torcaso et al., 2017). Although it is difficult to dissect a causal link between early binge-drinking history and the manifestation of mental disorders in later life, support for a direct cause–effect relationship can be derived from the animal literature (Koob, 2014; Becker, 2017) . As an example from our own laboratory, adult, male mice with a prior history of binge-drinking during adolescence exhibit both a hyper-anxious phenotype and augmented alcohol intake, relative to both water-drinking controls and mice with a more recent binge-drinking history during adulthood (Lee et al., 2017a). Further, in so far as we have investigated in male mice, the age-related differences in the temporal manifestation of withdrawal-induced negative affect reflect an interaction between the age of binge-drinking onset and time-dependent changes in the expression and function of glutamate receptor-related proteins within extended amygdala structures (Lee et al., 2016, 2017a, 2018a,b). Such data argue that, at least in male mice, adolescent-onset binge-drinking negatively impacts the development of the

mesocorticolimbic glutamate system within the major neurocircuits gating emotion and motivation.

Globally, a sex difference exists with respect to the prevalence of affective disorders, with females being twice as likely as males to be diagnosed with an anxiety-related disorder, irrespective of past or concurrent drug abuse (World Health Organization, 2014). The results of the extant literature focused on how biological sex and/or gender interacts with the age of drinking-onset to influence binge-drinking propensity/the development of AUD are less consistent, with data from the human literature pointing to sociocultural factors as major influences on study outcomes (Agabio et al., 2017; Becker et al., 2017; Wilsnack et al., 2018). In contrast, robust sex differences exist with respect to alcohol consumption in laboratory animals (including binge-drinking), with female non-human primates, rats, and mice consuming more alcohol than age-matched males in the majority of studies (Finn et al., 2010; Becker et al., 2017; Logrip et al., 2018). Female laboratory rodents also escalate alcohol-taking more quickly and exhibit greater relapse-like drinking than their male counterparts (Finn et al., 2010; Becker et al., 2017; Logrip et al., 2018). While fewer in number, some animal studies have examined the interactions between biological sex and age of drinking-onset with respect to binge-drinking-related outcomes (Strong et al., 2010; Melón et al., 2013; Cozzoli et al., 2014; Logrip et al., 2018; Szumlinski et al., 2019). Of direct relevance to this project, we demonstrated recently that akin to adult males (Lee et al., 2016), adult female B6 mice with a 2-week history of binge-drinking exhibit robust sings of negative affect during early (1 day) withdrawal (Szumlinski et al., 2019). However, in contrast to males (Lee et al., 2018a; Lees et al., 2019), this negative affective state persists in

adult females for at least 30 days post-drinking (Szumlinski et al., 2019). We also detected what might be a sex difference in the onset of a negative affective state during withdrawal in binge-drinking adolescents; female mice with a prior history of adolescent binge-drinking exhibit signs of a negative affective state in both early and later withdrawal (Szumlinski et al., 2019), while that observed in males incubates with the passage of time post-drinking (Lee et al., 2017a). Taken together, these findings regarding the interaction between sex and age of binge-drinking onset suggest that females may be more susceptible than males to developing a long-lasting change in emotionality during protracted alcohol withdrawal that warranted a direct investigation.

Herein, we examined sex differences in the effects of withdrawal from a 2-week binge-drinking history either during adolescence or adulthood upon anxiety- and depression-like behavior in B6 mice. As in our published work (Lee et al., 2016, 2017a; Szumlinski et al., 2019), a subset of mice was tested for anxiety- and depression-like behavior at one day post-drinking to test the hypothesis that adolescent female "bingers" will exhibit an earlier onset of negative affective signs, than their male binge-drinking counterparts. To extend the results of our prior studies using a 30-day withdrawal period (Lee et al., 2016, 2017a; Szumlinski et al., 2019), another subset of mice was tested for affective behavior at 70 days post-drinking. This was done to probe (1) the permanency of the effects of adolescent binge-drinking upon emotionality and (2) potential sex differences in the longevity of the binge-drinking effect in adult animals. Baseline and test-induced increases in plasma corticosterone (CORT) were examined to relate behavioral differences to the function of the hypothalamo–pituitary–adrenal (HPA) axis, based on evidence that sex- and age-related differences exist

with respect to HPA function that may have relevance for manifestation of binge-drinking, the etiology of AUD and affective disorder comorbidity (Moore et al., 2010; Melón et al., 2013; Romeo et al., 2016; Logrip et al., 2018; Romeo, 2018; Wellman et al., 2018).

2.2. Materials and Method

2.2.1. Subjects

This study employed a combination of male and female, adolescent (postnatal day PND = 28/29) and adult (PND = 56) C57BL/6J (B6) mice obtained from The Jackson Laboratory (Sacramento, CA, United States) or bred in-house in the Psychology vivarium at the University of California, Santa Barbara (UCSB) from breeder pairs originally obtained from the Jackson Laboratory. Mouse origin was based on practical considerations at the time of testing of the final cohorts of this large-scale study (fecundity of breeders, number of age-appropriate offspring available for testing) and was not considered to be a major factor in determining binge-drinking behavior, based off prior work conducted by our laboratory (Szumlinski et al., 2019). At PND 21, mice bred in-house were weaned from their litters and placed with different littermates of the same age and sex in groups of 4 in polycarbonate cages. As in our prior work (Lee et al., 2016, 2017a; Szumlinski et al., 2019), commercially sourced mice arrived at 21–22 days of age and were housed in same-age and -sex groups of 4. Mice were allowed 7 days to acclimate to a colony room, where they were housed under a reverse 12 h-light/dark cycle (lights off at 10:00 h) in a climate and humidity-controlled vivarium. The animals were identified using a combination of tail and ear markings and the mice from the two different sources were assigned to the different conditions as equally as

possible. Food and water were available ad libitum to all the animals except during the 2-h alcohol-drinking period. All the cages were lined with sawdust bedding and nesting materials in accordance with vivarium protocols. All routine cage cleaning/bedding changes were suspended at least 5 days prior to testing for behavioral signs of negative affect to eliminate potential confounds due to the change in the home-cage environment and foreign handling by the vivarium staff. All experimental procedures were in compliance with The Guide for the Care and Use of Laboratory Animals (2014) and approved by the Institutional Animal Care and Use Committee of the University of California, Santa Barbara.

2.2.2. Drinking-In-The-Dark (DID) Procedures

Approximately half of the mice ($N = 102$) were subjected to 14 consecutive days of binge- drinking using a multi-bottle-choice DID procedure that involved concurrent access to unsweetened ethanol at 5, 10, 20, and 40% (*v*/*v*) concentrations (Lee et al., 2016, 2017a; Szumlinski et al., 2019). On each drinking day, all animals were transferred into a dark, non-colony, procedural room within the vivarium, and the mice slated to binge-drink were transferred from their home cages to an individual drinking cage lined with sawdust bedding, situated on a free-standing rack and allowed to acclimate to the drinking cage for 1 h before bottle presentation (Szumlinski et al., 2019). After cage habituation, sipper tubes containing the alcohol solutions were placed on the drinking cage with the location of sipper tubes randomized daily and animals were allowed 2-h access (14:00–16:00 h). At 16:00 h, the sipper tubes were removed from the drinking cages and the binge-drinking mice were then transferred back into their home cages. To facilitate study throughput, water control mice remained in their home cages, but were placed onto the same free-standing rack as the binge-

drinking mice for the 3-h period. The water-drinking controls were not singly housed in this study, based on the collection of results from prior work, admittedly conducted exclusively in male mice, indicating comparable anxiety/depression-like behavior between water controls singly-housed for 3 h/day during the drinking procedures versus those merely transferred to the free-standing rack during this period (Lee et al., 2015, 2016, 2017a-b, 2018a-c). At the end of each drinking session, all the mice were returned the colony room. Mice were weighted every 3–4 days during the drinking procedures and their recorded weight was used in to calculate alcohol intake.

2.2.3. Blood Alcohol Concentration Sampling

Immediately after the 2-h alcohol-drinking period on the 10th drinking day, submandibular blood samples were collected from alcohol-drinking mice only. This sampling time-point was selected as the alcohol intake had stabilized, thereby providing a more accurate measure of their average daily alcohol consumption. Additionally, this time-point allowed sufficient recovery time prior to behavioral testing at the 1-day withdrawal time-point. A timeline of all the procedures employed in this study is provided in **Figure 2.1** below.

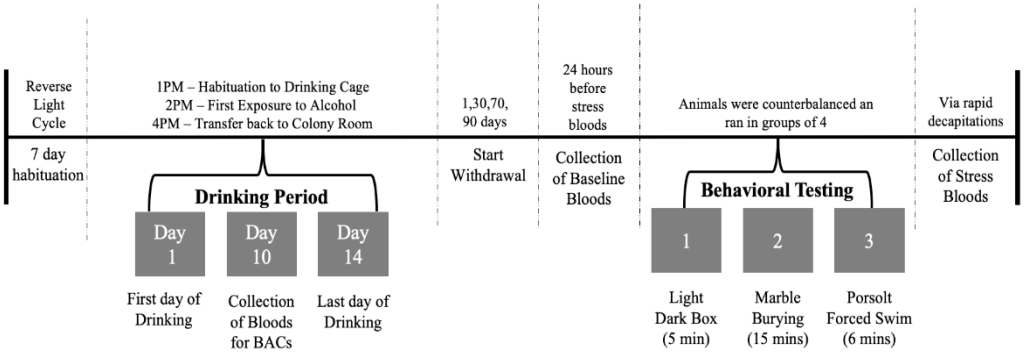


Figure 2.1. Summary of the procedural timeline for this study.

2.2.4. Gas Chromatography

Headspace gas chromatography is the gold standard for ethanol analysis due to its effectiveness and accuracy in determining levels in various substances, including blood (Tiscione et al., 2011). BACs were determined using a Shimadzu GC-2014 gas chromatography system (Shimadzu, Columbia, MD, USA) and the data was determined via the GC Solutions version 2.10.00 software. Samples were diluted at 1:9 with non-bacteriostatic saline (50 µL of sample). Acetone and dichloromethane were used as the pre-solvents due to their lower boiling point versus ethanol. Each sample was tested within 1-week of blood collection to reduce the potential for ethanol evaporation during storage. The determination of ethanol from each sample was derived using the standard curve equation determined prior to analyses of the samples. A new standard curve was formulated for each cohort of blood samples to ensure maximal accuracy. After the ethanol peak area was determined, the peak area was used to determine the ethanol concentration and subsequently the percent of ethanol in the blood. The BACs were then correlated with the alcohol intake observed on day 10 of drinking, which is when the blood was sampled.

2.2.5. Baseline and Stressor-Induced CORT Assay

To assay circulating plasma CORT levels, submandibular blood samples (50 µL) were collected from all the mice 24 h before behavioral testing for negative affect (for baseline CORT) at approximately 10:00 h (±30 min) and trunk blood was collected immediately following behavioral testing at approximately 17:00 h (±30 min) to index stressor-induced changes in circulating CORT. For all the animals tested at 1-day

withdrawal, the baseline blood samples were collected before mice were habituated to the drinking cages on the final (14th) day of drinking. All the bloods samples were collected in blood collection tubes lined with lithium heparin (BD Vacutainer, Mississauga, ON, Canada) and centrifuged at 10,500 rpm at 4 °C for 20-min to obtain plasma. The extracted plasma sample was kept frozen at −80 °C until assayed. Duplicate samples were analyzed using the DetectX Corticosterone Immunoassay kit K014-H5 (Arbor Assays, Ann Arbor, MI, USA). CORT levels were determined following the manufacturer's recommended instructions.

2.2.6. Behavioral Testing

In prior work, male B6 mice with a history of adolescent-onset binge-drinking exhibit no signs of negative affect when tested at one day withdrawal but exhibit robust anxiety- and depressive-like behaviors when tested at 30 days withdrawal (Lee et al., 2016, 2017a). In contrast, male B6 mice with a history of adult-onset binge-drinking exhibit signs of hyper-anxiety at 1-day withdrawal, but this negative affective state is no longer detectable at 30 days withdrawal (Lee et al., 2016, 2017a). Female B6 mice with a history of either adolescent- or adult-onset binge-drinking exhibit signs of anxiety- and depressive-like behaviors during both early and later withdrawal (Szumlinski et al., 2019). To directly examine for sex by age interactions in the effects of binge-drinking upon negative affect during alcohol withdrawal, we conducted a 1-day behavioral test battery consisting of the light–dark shuttle-box test, the marble burying test, and the Porsolt forced swim test. We and others have shown that these behavioral assays are sensitive to withdrawal-induced changes in negative affect, as well as age-related differences therein (Jacobson et al., 2007; McCall et al., 2013; Lee et al., 2016, 2017a; Szumlinski et al., 2019).

2.2.6.1. Light–Dark Shuttle-Box

The light–dark shuttle-box was used to measure anxiety-like behaviors, with decreased activity in the light-side interpreted as reflecting an anxiety-like phenotype (Crawley, 1985; Gallo et al., 2004). Animals were placed into a polycarbonate box measuring 46 cm in length × 22 cm in width × 24 cm in height. The box was divided into two environments, one side is white with no lid and the other side was black with a black lid (respectively, light versus dark side). During the experiment, the two environments were accessible through a central divider with an opening. The animals were first introduced to the dark environment by the experimenter and using AnyMaze™ tracking software (Stoelting Co., Wood Dale, IL, USA), our dependent measures of latency to enter the light side, total time spent in the light side and total number of light entries were recorded over a 5-min period. The boxes were cleaned in-between each use with Rescue Disinfectant Veterinary Wipes (Virox Animal Health, Oakville, ON, Canada). Immediately upon completion of this assay, the animals were transferred back into their home cages and transported to a distinct procedural room for marble-burying testing.

2.2.6.2. Marble-Burying Test

The marble-burying test is particularly sensitive to the effects of alcohol withdrawal based on our prior work (Lee et al., 2015, 2016, 2017a-b, 2018a-c; Szumlinski et al., 2019). Mice were placed in polycarbonate cage (12 cm × 8 cm × 6 cm), with 5-cm deep sawdust bedding on top of which marbles were arranged equidistantly. The percent of marbles buried (i.e., 75% covered by bedding) was determined after a 15-min session. The behavior of the

animals was also video-recorded using AnyMaze^{TM} tracking software and the total time spent burying and the latency to start burying was recorded by a blind observer using a stopwatch. At the end of the marble-burying session, the animals were returned to their home cages and transported to a 3rd procedural room for forced swim testing.

2.2.6.3. Porsolt Forced Swim Test

The Porsolt Forced Swim test is commonly employed assay for depressive-like behaviors and their reversal by anti-depressant treatments (Porsolt et al., 2001). Excessive swimming behavior in this assay can be reversed by pretreatment with anxiolytic medications and thus, has been used by our group as an additional measure of anxiety-like behavior (Strong et al., 2010; Cozzoli et al., 2014; Lee et al., 2018b). In our paradigm, an 11-cm diameter cylindrical glass container is filled with room temperature water and animals are tested over a 6-min period during which AnyMaze^{TM} tracking software determined the latency to first immobile episode, total time spent immobile, and the number of immobile episodes. Immobility is defined as the lack of vertical or horizontal displacement of the animal's center of gravity for at least 5-s. Upon the conclusion of this assay, animals were euthanized by rapid decapitation and trunk blood collected to index stressor-induced increases in plasma CORT.

2.2.7. Statistical Analysis

All statistical tests were conducted using the IBM SPSS Statistics software (IBM Corp. IBM SPSS Statistics for Macintosh, Version 24.0. Armonk, NY, USA). All graphs were created using the GraphPad Prism software (GraphPad Prism Software for Macintosh,

Version 8.01. La Jolla, CA, USA). Previous findings from our laboratory indicate an age-dependent effect of alcohol withdrawal upon anxiety (Lee et al., 2016, 2017a; Szumlinski et al., 2019). Thus, to increase the statistical power to detect lower level sex differences, the data for the Age and Withdrawal factors were analyzed separately using between-subjects univariate Analyses of Variance (ANOVAs). For all Sex × Drinking ANOVAs conducted on the data for affective behavior, $\alpha = 0.05$ was used and post-hoc *t*-test comparisons were employed when appropriate. Based on our prior evidence for age-related differences in the effects of binge-drinking upon anxiety-related behavior of male mice, we classified all behavioral ANOVAs rendering an $\alpha = 0.05 - 0.10$ as a statistical and notable trend (Lee et al., 2015, 2016, 2017a, 2018a-c). To ensure that the mice tested in early versus later withdrawal exhibited comparable alcohol intake, the average total alcohol intake over the 14-day drinking period was analyzed using an Age × Sex × Withdrawal ANOVA, with $\alpha = 0.05$. The CORT data were also analyzed separately for adolescent and adult mice using a Sex × Drinking × Withdrawal ANOVA with $\alpha = 0.05$. We normalized the stressor-induced changes in CORT to the baseline CORT levels for each subject and conducted a similar univariate analysis. Pearson's correlational tests were conducted to correlate: (1) BACs with alcohol intake; (2) alcohol intake with our CORT and behavioral measures; and (3) our CORT measures with behavior. As no a priori hypothesis regarding sex differences was established, $\alpha = 0.05$ was used for all CORT- and BAC-related analyses.

2.3. Results

2.3.1. Alcohol Intake and Blood Alcohol Concentrations

Binge-drinking animals consumed on average 4.52 ± 1.1 g/kg alcohol during the 14-day drinking period. The univariate Age × Sex × Withdrawal ANOVA resulted in no significant interactions (p's > 0.07; **Figure 2.2A**). However, we detected a main Age effect ($F(1,94) = 54.48$, $p < 0.001$), which reflected more alcohol consumption in adolescent versus adult mice (**Figure 2.2B**) and a main Sex effect ($F(1,94) = 34.33$, $p < 0.001$) that reflected more alcohol consumption in females versus males (**Figure 2.2C**). When all the animals were considered, a Pearson's correlation showed a positive relationship between BAC levels and alcohol intake ($r = 0.529$, $p < 0.0001$, **Figure 2.2D**), with a pattern of group differences in line with those observed for the total alcohol intake (**Figure 2.2E** vs. **Figure 2.2A**).

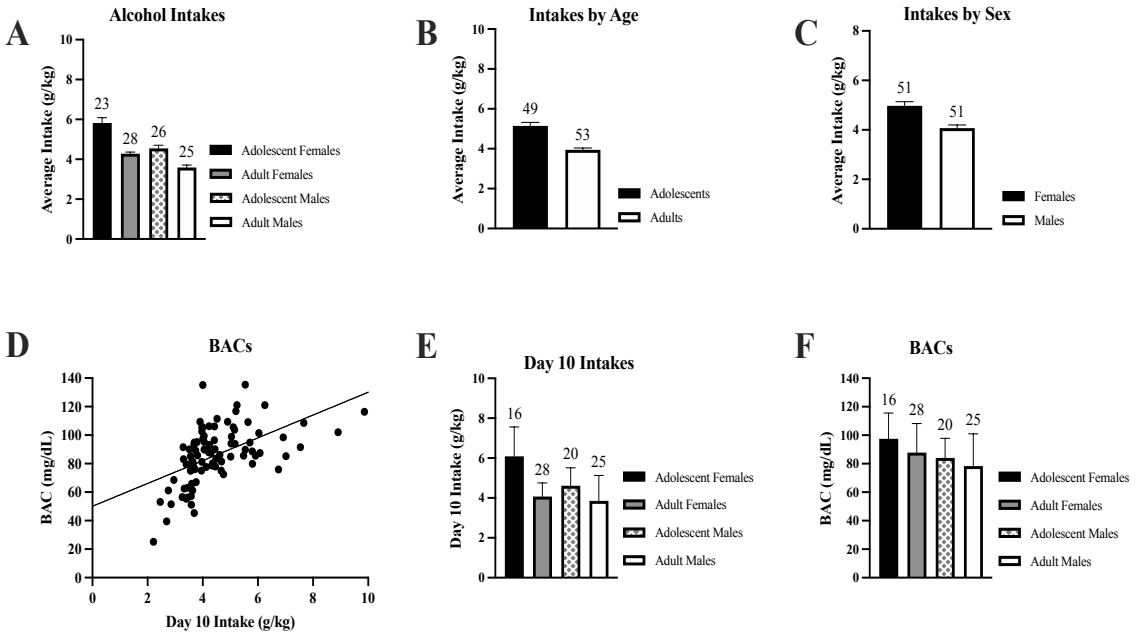


Figure 2.2. Summary of group differences in alcohol intake under our Drinking-in-the-Dark (DID) procedures. (**A**) Comparison of the average alcohol intake (g/kg) of the adult and adolescent male and female mice over the course of the 14-day drinking period. (**B**) Data from 1a, collapsed across Sex to illustrate the main Age effect detected by ANOVA. (**C**) Data from 1a, collapsed across Age to illustrate the main Sex effect detected by ANOVA. (**D**) Correlation between alcohol intake and BACs collected on Day 10 of drinking. (**E**) Comparison of group differences in alcohol intake on Day 10 of drinking when

blood was sampled. (**F**) Comparison of group differences in the average BACs attained on Day 10 of drinking. The data represent the means ± SEMs for the numbers of mice indicated in each panel.

2.3.2. Light–Dark Shuttle-Box

2.3.2.1. Latency to Enter the Light Side

A Sex × Drinking ANOVA conducted on the latency to enter the light side of the shuttle-box yielded no significant interaction for the adolescent mice in early withdrawal (**Figure 2.3A**) ($F(1,43) = 0.01$, $p = 0.91$). However, a significant Sex × Drinking interaction was observed for the adult-onset animals tested in early withdrawal (**Figure 2.3B**) ($F(1,51) = 7.53$, $p = 0.01$). Deconstruction of this interaction did not reveal any significant group differences; however, a statistical trend in the latency to enter the light side was observed for female mice in that female binge-drinkers exhibited a shorter latency to enter the light side, compared with their water-drinking counterparts ($t(26) = 1.97$, $p = 0.06$). In contrast, male binge-drinkers exhibited a longer latency to enter the light side than their water-drinking counterparts (t (25) = 1.92, $p = 0.07$).

For adolescent mice in protracted withdrawal, no significant Sex × Drinking interactions were observed (**Figure 2.3C**) ($F(1,36) = 0.01$, $p = 0.93$). However, a significant main effect of drinking was noted for these animals ($F(1,36) = 6.29$, $p = 0.02$), reflecting a shorter latency to enter the light side for the adolescent binge- versus water-drinking animals. No significant Sex x Drinking interaction or main effects were confirmed for the adult mice in protracted withdrawal (**Figure 2.3D**) ($F(1,37) = 1.23$, $p = 0.27$).

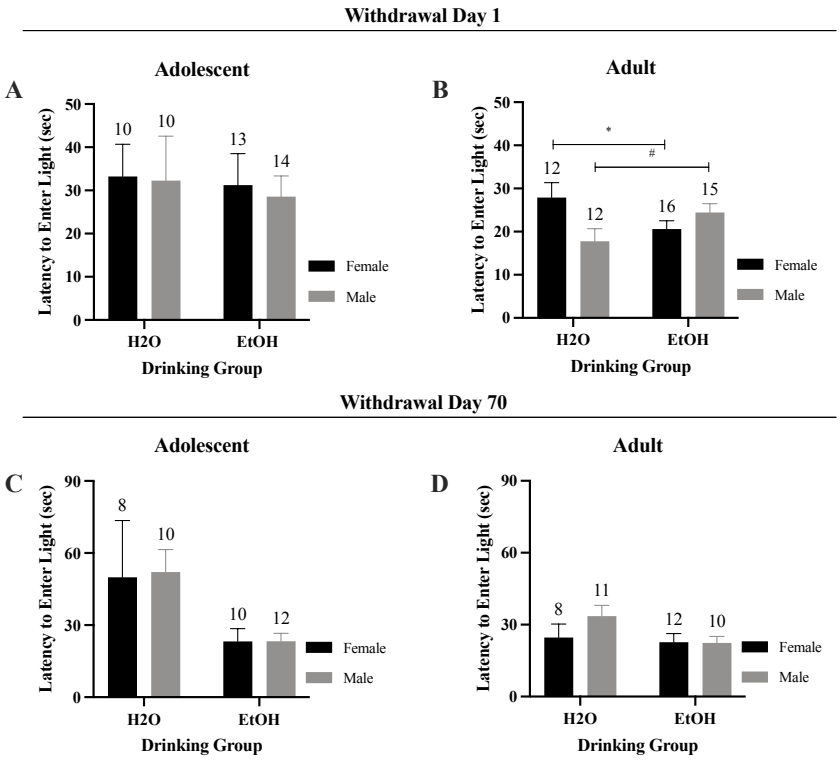


Figure 2.3. Summary of the results of the Sex × Drinking ANOVA conducted on the data for the latency to enter the light-side of the light–dark shuttle-box. (**A**) Depiction of the results for the adolescent-onset mice at 1-day withdrawal. (**B**) Depiction of the Sex × Drinking interaction observed for the adult-onset mice at 1-day withdrawal. (**C**) Depiction of the results for the adolescent-onset mice at 70-days withdrawal. (**D**) Depiction of the results for the adult-onset mice at 70-days withdrawal. The data represent the means ± SEMs for the numbers of mice indicated in each panel. * $p < 0.05$ for female H2O vs. female EtOH; # $p <$ 0.05 for male H2) vs. male EtOH.

2.3.2.2. Time Spent in the Light Side

A Sex x Drinking ANOVA indicated no significant interaction for the total time spent in the light side of the shuttle-box by the adolescent-onset mice tested in early withdrawal (**Figure 2.4A**) ($F(1,43) = 0.93$, $p = 0.34$). However, a significant main effect of Drinking was observed for these animals ($F(1,43) = 22.54$, $p < 0.001$), which reflected more time spent in the light-side by the binge-drinkers versus the water controls. For the adult-onset mice tested in early withdrawal, the interaction exhibited a statistical trend (**Figure 2.4B)** ($F(1,51) =$

3.50, $p = 0.07$), which reflected more time spent in the light-side by alcohol- versus water-drinking males (t (25) = 1.95, $p = 0.06$). In contrast, no significant alcohol-related trend was observed for female mice tested in early withdrawal (t-test: $p = 0.51$).

Analyses of the total time spent in the light side for adolescent mice tested in protracted withdrawal also revealed a Sex x Drinking trend (**Figure 2.4C**) (F(1,36) = 3.17, $p = 0.08$). In adolescent males, binge-drinkers spent significantly more time in the light-side compared to their water-drinking controls (t (20) = 4.13, $p = 0.001$). In contrast, no effect of Drinking was observed for the adolescent females tested in protracted withdrawal (t-test, $p = 0.58$). No significant two-way interaction or trend was observed for the adult-onset mice tested in protracted withdrawal (**Figure 2.4D**) (F(1,37) = 1.84, $p = 0.18$). However a significant main effect of Drinking was detected in the adult animals (F(1,37) = 8.44, p = 0.01), with adult binge-drinkers spending more time in the light side versus the adult water controls.

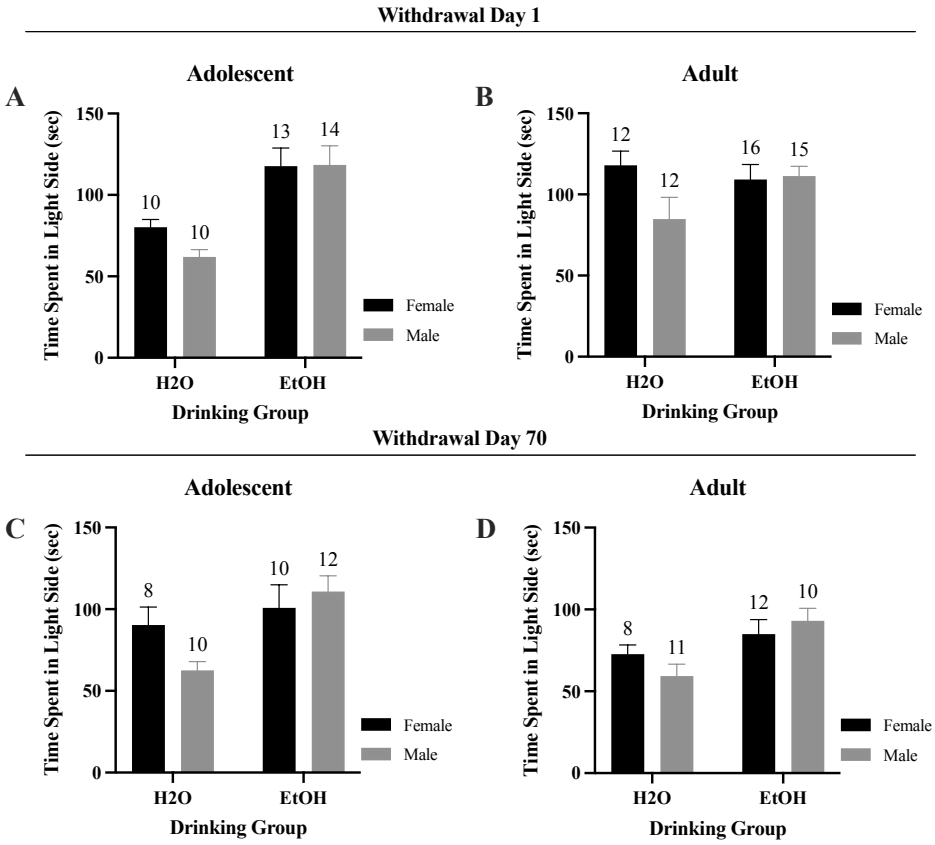


Figure 2.4. Summary of the results for the Sex × Drinking ANOVA observed for the total time spent in the light-side in the light–dark shuttle-box test. (**A**) Depiction of the results for the adolescent-onset mice at 1-day withdrawal. (**B**) Depiction of the results observed for the adult-onset mice at 1-day withdrawal. (**C**) Depiction of the results for the adolescent-onset mice at 70-days withdrawal. (**D**) Depiction of the results for the adult-onset mice at 70-days withdrawal. The data represent the means ± SEMs for the numbers of mice indicated in each panel.

2.3.2.3. Number of Light Entries

Examination of the total number of light entries in early withdrawal indicated no significant interaction or trends for either the adolescent-onset (**Figure 2.5A**) ($F(1,43) = 0.76$, $p = 0.39$) or the adult-onset mice (**Figure 2.5B**) ($F(1,51) = 0.09$, $p = 0.76$). However, a trend for a main effect of Sex was observed for the adolescent mice tested in early withdrawal ($F(1,43) = 2.98$, $p = 0.09$), that reflected more light entries in female versus male mice.

For the animals in protracted withdrawal, no significant interaction or trend was observed for the adolescent mice (**Figure 2.5C**) ($F(1,36) = 0.38$, $p = 0.54$) or for the adult mice (**Figure 2.5D**) ($F(1,37) = 0.80$, $p = 0.38$); however a significant main effect of Drinking was noted for the adult-onset mice ($F(1,37) = 13.65$, $p = 0.001$) indicating that the binge-drinkers had a higher number of light entries than water-controls.

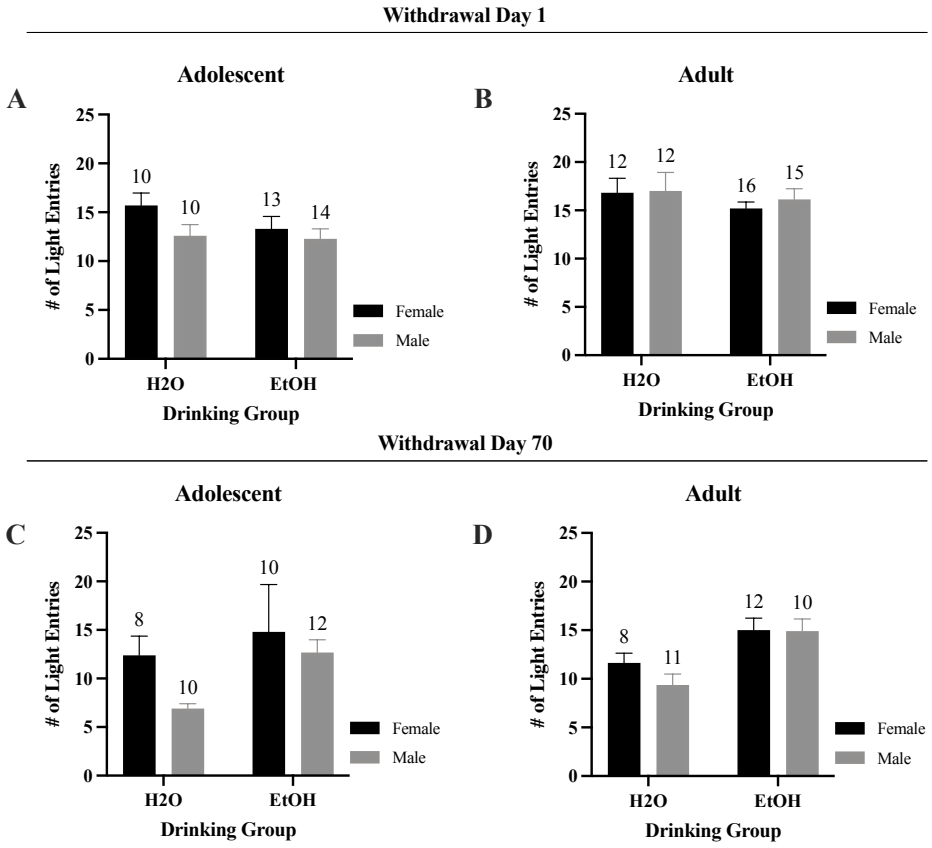


Figure 2.5. Summary of the results for the Sex × Drinking ANOVA observed for the total number of entries to the light-side in the light–dark shuttle-box test. (**A**) Depiction of the results for the adolescent-onset mice at 1-day withdrawal. (**B**) Depiction of the results observed for the adult-onset mice at 1-day withdrawal. (**C**) Depiction of the results for the adolescent-onset mice at 70-days withdrawal. (**D**) Depiction of the results for the adult-onset mice at 70-days withdrawal. The data represent the means ± SEMs for the numbers of mice indicated in each panel.

2.3.3. Marble Burying Test

2.3.3.1. Latency to Start Burying

Examination of the latency to start burying for adolescent-onset mice in early withdrawal indicated no significant Sex × Drinking interaction (**Figure 2.6A**) ($F(1,43) = 1.58$, $p = 0.22$). However, there was a significant main effect of Drinking ($F(1,43) = 8.11$, $p = 0.01$), which reflected a shorter latency to start burying in alcohol- versus water-drinking adolescents. For the adult-onset mice, the results of the two-way ANOVA indicated a statistical trend for the interaction (**Figure 2.6B**) ($F(1,51) = 3.76$, $p = 0.06$), which reflected a shorter latency to bury in alcohol- versus water-drinking males tested in early withdrawal ($t(25) = 2.30$, $p = 0.03$), but no alcohol-related difference in adult females. ($p = 0.92$).

The Sex x Drinking ANOVA for the adolescent mice tested in protracted withdrawal indicated a significant interaction (**Figure 2.6C**) ($F(1,36) = 4.38$, $p = 0.04$). Deconstruction of this interaction along the Sex factor revealed a shorter latency to bury in alcohol- versus water-drinking females ($t(16) = 5.29$, $p < 0.001$) and males ($t(20) = 4.90$, $p < 0.001$). A two-way interaction was not observed for the adult-onset mice tested in protracted withdrawal (**Figure 2.6D**) ($F(1,37) = 0.65$, $p = 0.43$). However, a significant main effect of Drinking Group was apparent that reflected a shorter bury latency in binge-drinking mice versus water-drinking counterparts ($F(1,37) = 34.76$, $p < 0.001$).

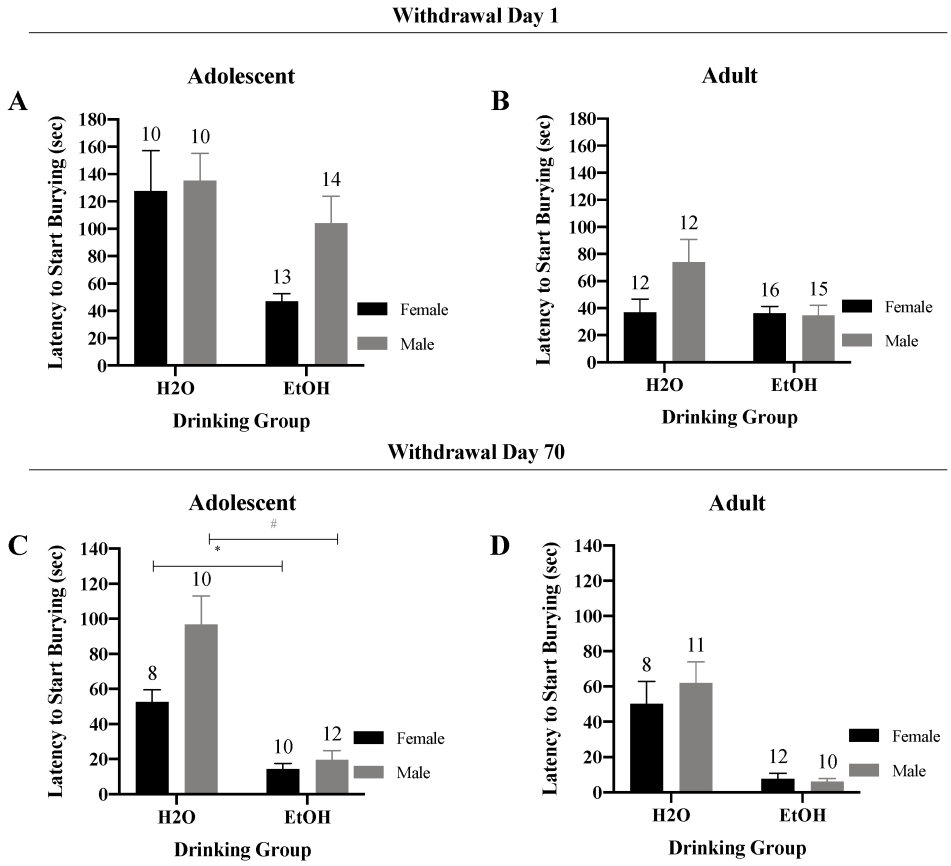


Figure 2.6. Summary of the results for the Sex × Drinking ANOVA observed for the latency to start burying marbles in the marble-burying test. (**A**) Depiction of the results for the adolescent-onset mice at 1-day withdrawal. (**B**) Depiction of the results observed for the adult-onset mice at 1-day withdrawal. (**C**) Depiction of the Sex × Drinking interaction for the adolescent-onset mice at 70-days withdrawal. (**D**) Depiction of the results for the adult-onset mice at 70-days withdrawal. The data represent the means ± SEMs for the numbers of mice indicated in each panel. * $p < 0.05$ for female H2O vs. female EtOH; # $p < 0.05$ for male H2) vs. male EtOH.

2.3.3.2. Time Spent-Burying

A Sex × Drinking ANOVA conducted on the time-spent burying marbles by adolescent-onset mice tested in early withdrawal indicated no significant interactions or noteworthy trends (**Figure 2.7A**) ($F(1,43) = 1.64$, $p = 0.21$). Similarly, the results of the two-way ANOVA for the adult-onset mice in early withdrawal uncovered no significant

interactions or trends were detected for the adult-onset mice tested in early withdrawal (**Figure 2.7B**) ($F(1,51) = 0.20$, $p = 0.65$). No observable main effects were found for either age group for this variable (p's > 0.05).

In protracted withdrawal., analyses of the data for adolescent-onset mice revealed a significant main effect of both Sex ($F(1,36) = 11.27$, $p = 0.002$) and Drinking Group ($F(1,36) = 6.43$, $p = 0.02$), but no significant Sex × Drinking interaction (**Figure 2.7C**) ($F(1,36) = 0.02$, $p = 0.88$). Female adolescent-onset mice spent more time burying in protracted withdrawal than males, with binge-drinking mice spending more time burying than water controls (**Figure 2.7C**). Analyses of the data for adult-onset mice did not yield a significant Sex × Drinking interaction nor any other notable trends for this variable (**Figure 2.7D**) ($F(1,37) = 1.72$, $p = 0.20$).

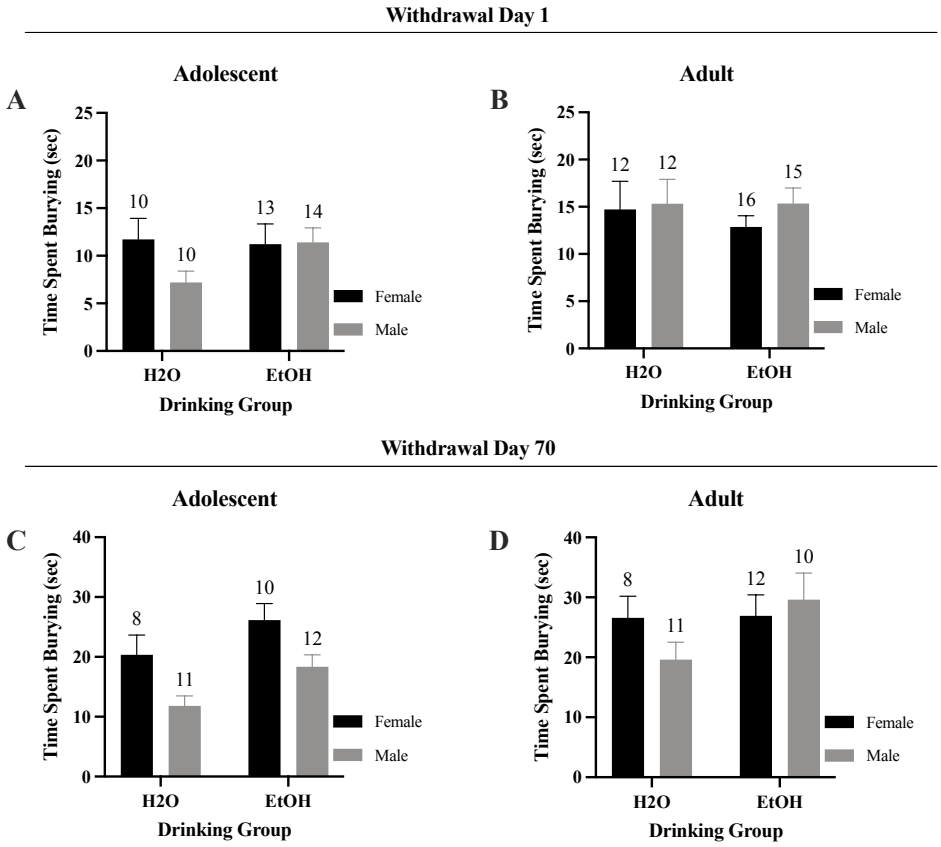


Figure 2.7. Summary of the results for the Sex × Drinking ANOVA observed for the total time spent burying marbles in the marble-burying test. (**A**) Depiction of the results for the adolescent-onset mice at 1-day withdrawal. (**B**) Depiction of the results observed for the adult-onset mice at 1-day withdrawal. (**C**) Depiction of the results for the adolescent-onset mice at 70-days withdrawal. (**D**) Depiction of the results for the adult-onset mice at 70-days withdrawal. The data represent the means ± SEMs for the numbers of mice indicated in each panel.

2.3.3.3. Percent of Marbles Buried

A Sex × Drinking ANOVA indicated no significant interactions for either the adolescent-onset (**Figure 2.8A**) ($F(1,43) = 0.004$, $p = 0.95$) or adult-onset (**Figure 2.8B**) ($F(1,51) = 0.16$, $p = 0.69$) mice in early withdrawal. Similarly, no significant interaction was detected for the adolescent-onset mice in later withdrawal (**Figure 2.8C**) ($F(1,36) = 0.10$, $p = 0.75$); although there was a significant main effect of Sex ($F(1,36) = 5.10$, $p = 0.03$), which

reflected a higher percentage of marble buried in the adolescent females versus males tested during protracted withdrawal. Results for the adult-onset mice tested in later withdrawal yielded a statistical trend for a Sex × Drinking interaction (**Figure 2.8D**) ($F(1,37) = 3.44$, $p = 0.07$). This trend reflected more marble-burying in male binge-drinkers versus their water controls (t (19) = 3.61, $p = 0.002$), while no alcohol-water difference was apparent in the females tested at this time (t-test, $p = 0.57$).

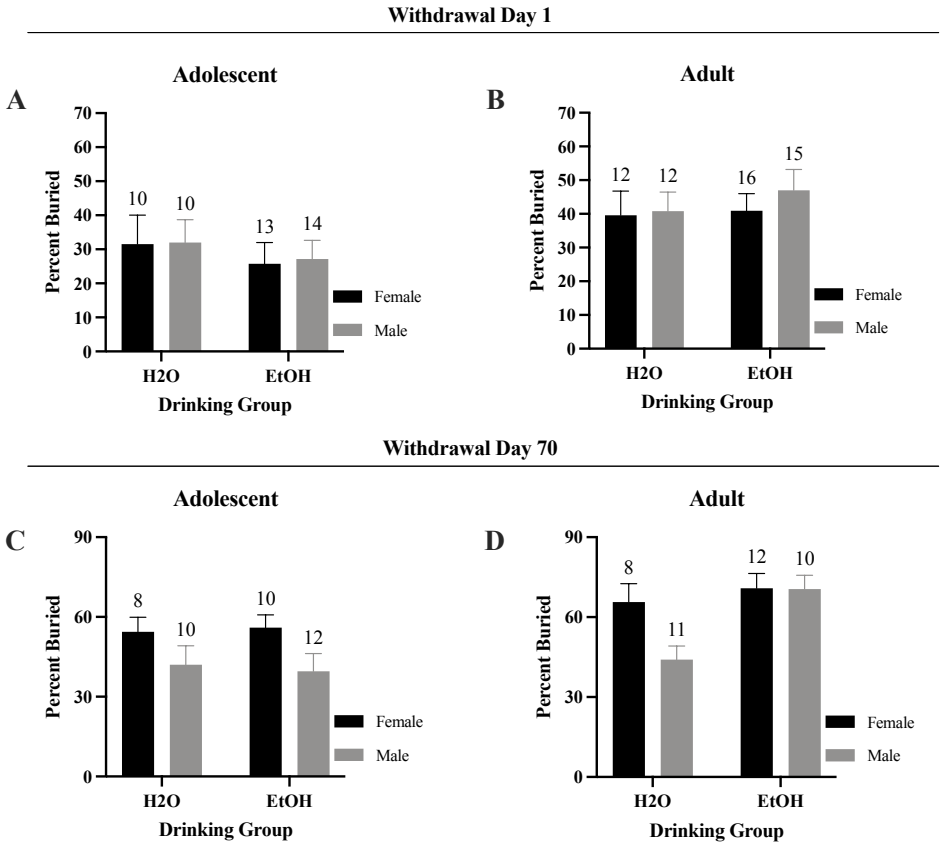


Figure 2.8. Summary of the results for the Sex × Drinking ANOVA observed for the percent of marbles buried. in the marble-burying test. (**A**) Depiction of the results for the adolescent-onset mice at 1-day withdrawal. (**B**) Depiction of the results observed for the adult-onset mice at 1-day withdrawal. (**C**) Depiction of the results for the adolescent-onset mice at 70-days withdrawal. (**D**) Depiction of the results for the adult-onset mice at 70-days withdrawal. The data represent the means ± SEMs for the numbers of mice indicated in each panel.

2.3.4. Porsolt Forced Swim Test

2.3.4.1. Latency to Immobility/Floating

In early withdrawal, a Sex × Drinking ANOVA conducted on the latency to first float by adolescent-onset mice indicated no significant interaction (**Figure 2.9A**) ($F(1,43) = 0.54$, $p = 0.47$); however, there was a significant main effect of Drinking Group ($F(1,43) = 8.06$, $p = 0.01$) that reflected a shorter latency to float in water- versus binge-drinking adolescents. Conversely, a significant Sex x Drinking interaction was detected for the adult-onset mice tested in early withdrawal (**Figure 2.9B**) ($F(1,51) = 5.56$, $p = 0.02$). Deconstruction of this interaction along the Sex factor revealed a shorter latency to float in binge-drinking versus water-drinking females (t (26) = 2.38, $p = 0.03$).

In contrast, no significant alcohol effect was noted for males (t-test, $p = 0.36$). In later withdrawal, no significant Sex × Drinking interaction was uncovered for either the adolescent-onset (**Figure 2.9C**) ($F(1,36) = 0.02$, $p = 0.88$) or adult-onset mice (**Figure 2.9D**) ($F(1,37) = 0.04$, $p = 0.84$). However, a significant main effect of Drinking was detected for the adult-onset mice, which reflected a longer latency to float in binge-drinking versus water controls ($F(1,37) = 5.56$, $p = 0.02$).

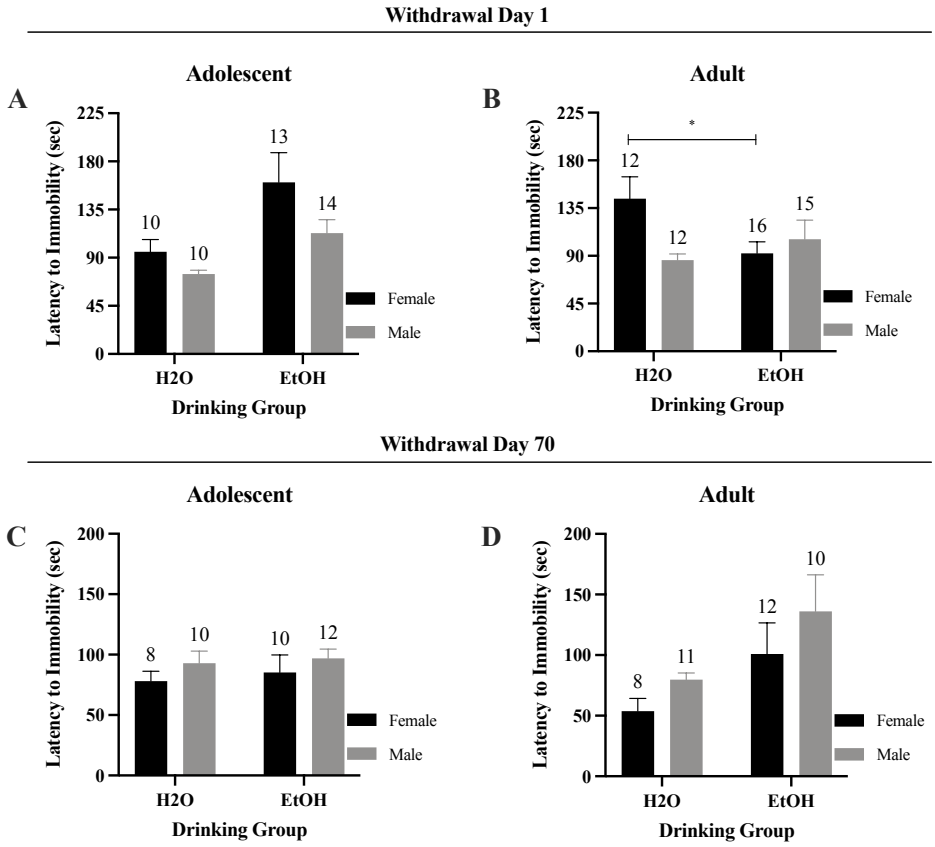


Figure 2.9. Summary of the results for the Sex × Drinking ANOVA observed for the latency to immobility in the Porsolt Forced Swim Test. (**A**) Depiction of the results for the adolescent-onset mice at 1-day withdrawal. (**B**) Depiction of the Sex × Drinking interaction detected for the female adult-onset mice at 1-day withdrawal. (**C**) Depiction of the results for the adolescent-onset mice at 70-days withdrawal. (**D**) Depiction of the results for the adult-onset mice at 70-days withdrawal. The data represent the means ± SEMs for the numbers of mice indicated in each panel. * $p < 0.05$ for female H2O vs. female EtOH.

2.3.4.2. Time Spent Immobile

Analyses of the time spent immobile during the forced swim test for the adolescent-onset mice tested in early withdrawal indicated no significant interaction (**Figure 2.10A**) ($F(1, 43) = 0.77$, $p = 0.39$), but significant main effects of both Sex ($F(1,43) = 8.61$, $p = 0.01$) and Drinking ($F(1,43) = 68.36$, $p < 0.001$). Overall, binge-drinking adolescents spent less time immobile versus water-drinking controls, with female mice spending less time immobile

than their male counterparts. In contrast, no significant interactions ($F(1,51) = 0.46$, $p = 0.50$) or main effects (p's > 0.05) were observed for the adult-onset mice tested in early withdrawal (**Figure 2.10B**).

In protracted withdrawal, the two-way ANOVA also failed to reveal a significant interaction for either the adolescent-onset (**Figure 2.10C**) ($F(1,36) = 0.00$, $p = 1.00$) or the adult-onset mice (**Figure 2.10D**) ($F(1,37) = 0.86$, $p = 0.36$). However, binge-drinking adult mice did spend significantly less time immobile than their water controls when tested in later withdrawal (Drinking effect: $F(1,37) = 6.99$, $p = 0.01$).

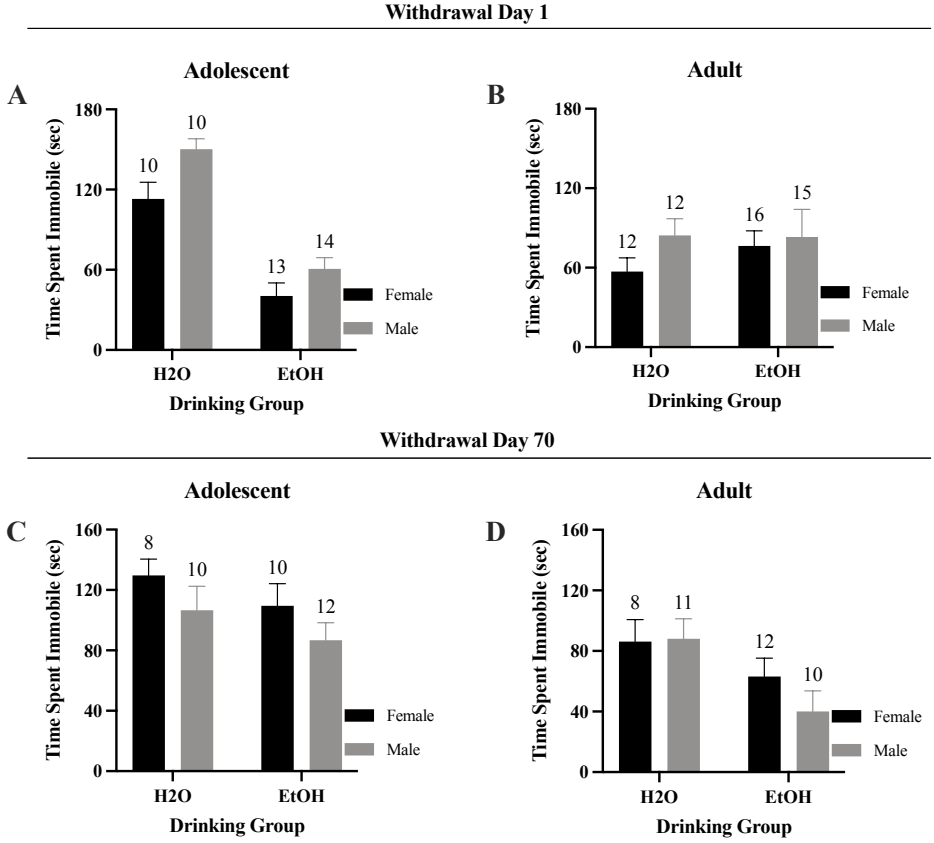


Figure 2.10. Summary of the results for the Sex × Drinking ANOVA observed for the total time spent immobile in the Porsolt Forced Swim Test. (**A**) Depiction of the results for the adolescent-onset mice at 1-day withdrawal. (**B**) Depiction of the results for the adult-

onset mice at 1-day withdrawal. (**C**) Depiction of the results for the adolescent-onset mice at 70-days withdrawal. (**D**) Depiction of the results for the adult-onset mice at 70-days withdrawal. The data represent the means ± SEMs for the numbers of mice indicated in each panel.

2.3.4.3. Immobile Episodes

In line with the data for the time spent immobile (**Figure 2.10A**), the data did not indicate a significant interaction for adolescent mice tested in early withdrawal (**Figure 2.11A**) ($F(1,43) = 0.02$, $p = 0.89$), but did show a significant main effect of both Sex ($F(1,43) = 4.70$, $p = 0.04$) and Drinking ($F(1,43) = 25.35$, $p < 0.001$), with binge-drinking mice exhibiting fewer immobile episodes than water-drinkers and female mice exhibiting less immobility than males. Interestingly, a notable trend for an interaction between Sex and Drinking Group was detected for the adult-onset mice tested in early withdrawal (**Figure 2.11B**) ($F(1,51) = 3.34$, $p = 0.07$). However, upon further analyses, no significant water-alcohol differences were observed for either female (t-test, $p = 0.36$) or male mice (t-test, $p = 0.12$).

For the animals in protracted withdrawal, the two-way ANOVA failed to determine a significant Sex × Drinking interaction for either adolescent-onset (**Figure 2.11C**) ($F(1,36) = 0.57$, $p = 0.45$) or adult-onset mice (**Figure 2.11D**) ($F(1,37) = 0.49$, $p = 0.49$). Nevertheless, a significant main effect of Drinking Group for the adult mice showed a higher number of immobile episodes for the water-drinking mice compared the binge-drinking adult mice in protracted withdrawal ($F(1,37) = 6.28$, $p = 0.02$).

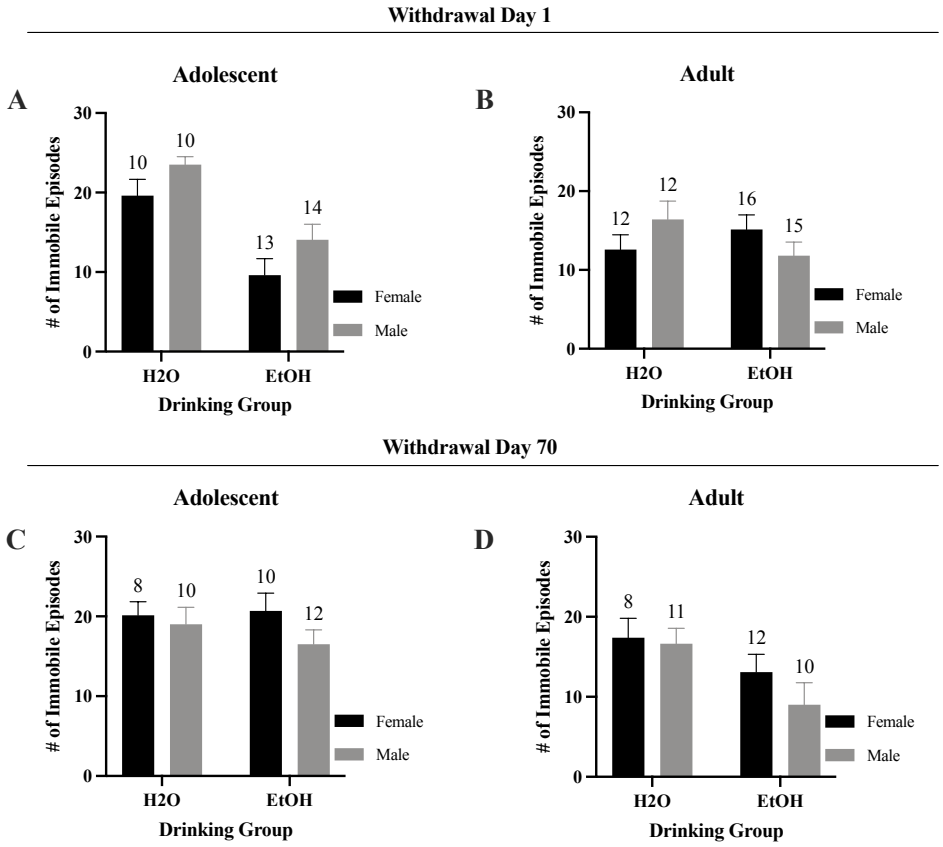


Figure 2.11. Summary of the results for the Sex × Drinking ANOVA observed for the total number of immobile episodes in the Porsolt Forced Swim Test. (**A**) Depiction of the results for the adolescent-onset mice at 1-day withdrawal. (**B**) Depiction of the results for the adult-onset mice at 1-day withdrawal. (**C**) Depiction of the results for the adolescent-onset mice at 70-days withdrawal. (**D**) Depiction of the results for the adult-onset mice at 70-days withdrawal. The data represent the means ± SEMs for the numbers of mice indicated in each panel.

2.3.5. Corticosterone Assay

2.3.5.1. Basal Corticosterone

Analyses of the basal CORT levels of the adolescent-onset mice indicated a significant Sex × Drinking × Withdrawal interaction ($F(1,82) = 4.77$, $p = 0.032$). Thus, the interaction was deconstructed along the Withdrawal factor to examine for Sex × Drinking interactions at each withdrawal time-point. This deconstruction revealed no significant

interaction for the adolescent mice tested on Withdrawal Day 1 ($p = 0.096$) or 70 ($p = 0.170$). The significant Sex × Drinking × Withdrawal interaction was then deconstructed along the Drinking factor to examine for withdrawal-dependent changes in basal CORT. In binge-drinking mice, a significant Sex × Withdrawal interaction was observed ($F(1,48) = 6.86$, $p = 0.012$), which reflected a withdrawal-dependent increase in basal CORT in the adolescent-onset female binge-drinkers (**Figure 2.12A**) (t (20) = 3.08, $p = 0.006$), but not in the other adolescent-onset groups (for female water, male water and male alcohol, t-tests, p's > 0.05). In contrast to the adolescent-onset mice, no group differences in basal CORT levels were detected in adult animals (**Figure 2.12B**) ($F(1,102) = 1.79$, $p = 0.184$).

2.3.5.2. Stressor-Induced Corticosterone

A Sex × Withdrawal × Drinking ANOVA indicated no significant interactions for stressor-induced CORT levels in adolescent-onset mice ($F(1,82) = 0.54$, $p = 0.47$). However, a main effect of Sex was detected ($F(1,82) = 65.33$, $p < 0.001$), which reflected lower stressor-induced CORT levels in females versus males (**Figure 2.12C**). For the adult-onset animals (**Figure 2.12D**), the 3-way ANOVA yielded an insignificant interaction ($F(1,102) = 0.703$, $p = 0.404$) and resulted in no significant main effects (all p's > 0.05).

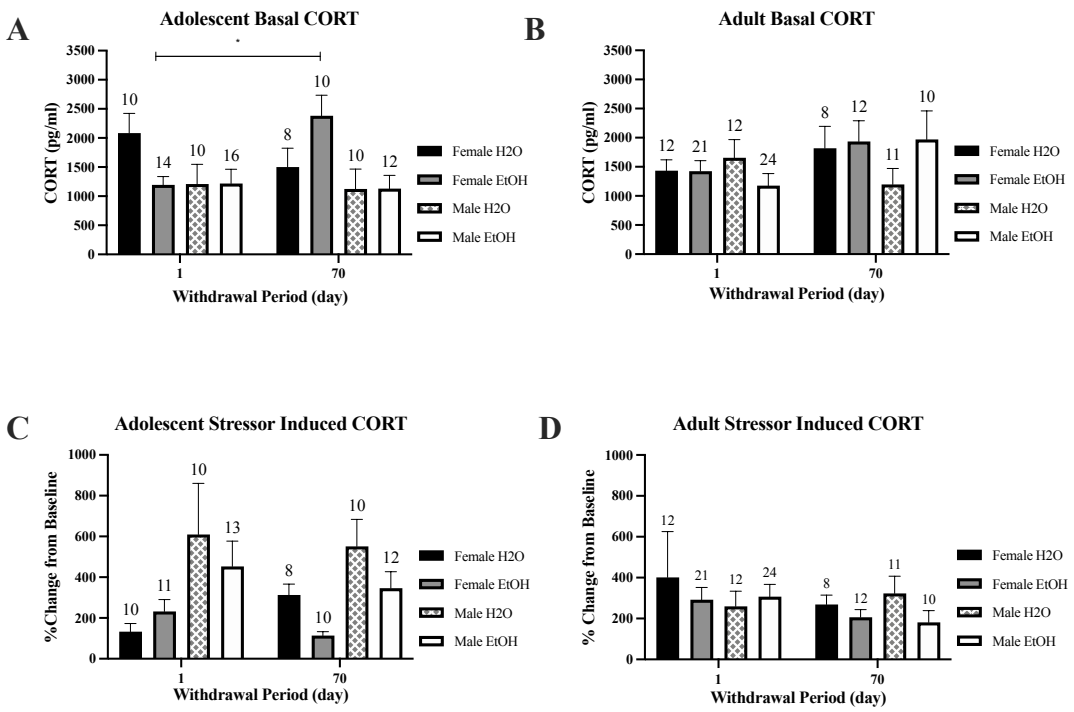


Figure 2.12. Summary of the subject factor interactions observed regarding plasma corticosterone (CORT). (**A**) Depiction of the Sex × Drinking × Withdrawal interaction observed for adolescent-onset drinking mice versus (**B**) the lack thereof for adult-onset drinking animals. (**C**) Depiction of the Sex × Drinking × Withdrawal interaction for stressor-induced corticosterone levels in adolescent-onset mice. (**D**) Depiction of the lack of group differences in stressor-induced corticosterone levels in adult-onset animals. The data represent the means ± SEMs for the numbers of mice indicated in each panel. * $p < 0.05$ WD1 vs. WD70 for female EtOH.

2.3.5.3. Inter-Relations between Alcohol Intake and Corticosterone Levels

When all mice were considered ($N = 197$), we did not find any significant correlations between the average alcohol intake of the mice and either basal CORT levels ($r = 0.003$, $p = 0.974$) or stressor-induced increases in CORT levels on the test day ($r = -0.024$, $p = 0.79$). As it might be predicted that alcohol consumption would have a greater impact upon basal and stressor-induced changes in CORT during early versus later withdrawal, we deconstructed the data along the Withdrawal factor for re-analysis. However, we still failed

to detect significant relationships between alcohol intake and basal or stressor-induced changes in CORT, even at 1-day withdrawal (r's < 0.51, p's > 0.25; data not shown). Given the sex- and age-related differences in alcohol intake (**Figure 2.2**), we also deconstructed the data along these subject factors and conducted additional follow-up correlational analyses to determine whether or not sex- or age-related differences might exist for the inter-relationship between alcohol intake and our CORT measures. The only significant relationship that was detected was a positive one between alcohol intake and stressor-induced CORT in males (r (64) = 0.322, p = 0.001). The remainder of the results failed to indicate any significant relationships between alcohol consumption and CORT, even when only the data from withdrawal day 1 were considered (data not shown).

2.3.5.4. Inter-Relations between Corticosterone Levels and Behavioral Indices of Negative Affect

When all mice were considered, we did detect significant correlations between basal CORT levels and both the total number of light entries from the light dark box test (**Figure 2.13A**) (r (177) = 0.211, p = 0.005) and the total time spent marble-burying (**Figure 2.13B)** (r (177) = 0.25, p = 0.001). The total time spent marble-burying was also inversely correlated with stressor-induced CORT (**Figure 2.13C**) (r (177) = −0.18, p = 0.01). However, inspection of **Figure 2.13C** suggested that this correlation may be driven by two mice with very high stressor-induced CORT responses. Indeed, analysis indicated that the data for these two animals were two standard deviations above the mean of the population. Thus, the data from these two mice were removed and re-analysis revealed instead a strong statistical trend for a correlation (**Figure 2.13C'**) (r (175) = 0.14, p = 0.057). When all mice were considered,

no other significant correlations were detected vis-à-vis the average total alcohol intake and our behavioral measures of negative affect (data not shown).

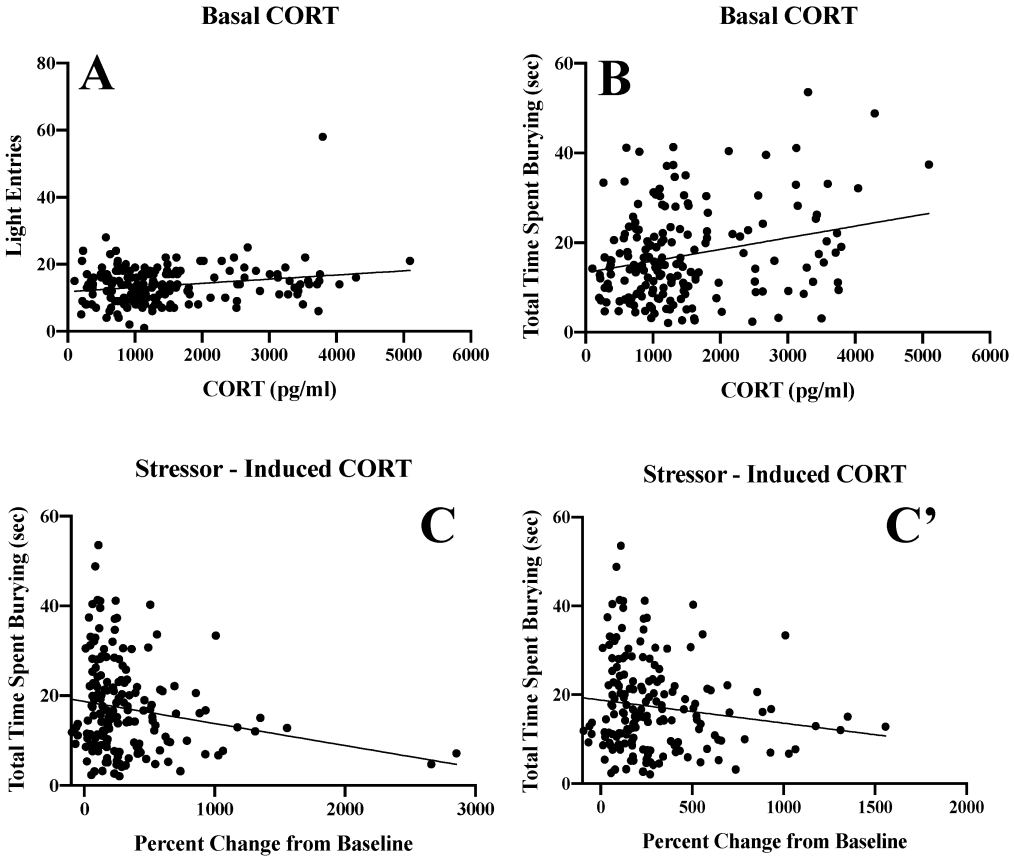


Figure 2.13. Summary of the significant inter-relations between corticosterone levels and behavioral indices of negative affect. A predictive relationship was observed between basal corticosterone (CORT) and (**A**) the number of light entries in the light–dark shuttle-box test and (**B**) the total time spent burying in the marble-burying test. (**C**) In contrast, when all animals were considered, an inverse relationship was observed between the stressor-induced changes in corticosterone and the time spent burying. (**C'**) Re-analysis of the data in Panel C following removal of the two outlier mice exhibiting very high stressor-induced CORT responses reduced the strength of this inverse relationship.

Although alcohol intake was found to be unrelated to either basal or stressor-induced CORT levels (see Section 2.3.5.3), we tested the possibility that the relationship between

CORT and behavior might vary as a function of binge-drinking history by deconstructing the data along the Drinking factor prior to re-analysis. However, the results of this re-analysis failed to indicate any correlations that were specific to the alcohol-drinking mice (**Table 2.1**).

We also examined how our subject factors of Sex and Age might influence the relationship between basal and stressor-induced changes in CORT and our behavioral measures of negative affect. When all mice were considered, basal CORT levels predicted the time spent burying, a relationship that held only when females were included in the analysis (r (86) = 0.28, p = 0.01), although a strong positive trend was also observed when only males were considered (r (93) = 0.20, p = 0.055). The relationship also held up when only adults were examined (r (94) = 0.28, p = 0.007; for adolescents, r (85) = 0.20, p = 0.07), arguing that the positive correlation reflected primarily the results of the adult mice. When all mice were considered, stressor-induced CORT levels were inversely related to the time spent burying (**Table 2.1**). While this correlation did not hold upon deconstruction along the Sex and Age factors, it is noteworthy that the relationship between these variables trended strongly in female mice (r (86) = −0.20, p = 0.07; for males, r(91) = −0.15, p = 0.15) and adolescent mice (r(85) = −0.20, p = 0.07; for adults: r(94) = −0.14, p = 0.17).

Drinking	Dependent	Basal CORT	Stressor-Induced
Water (N = 81)	Latency to Bury	r = 0.155, p = 0.168	r = −0.137, p = 0.223
	Time in light	r = 0.065, p = 0.562	r = 0.041, p = 0.717

Drinking	Dependent	Basal CORT	Stressor-Induced
	Light Entries	***r* = 0.229, *p* = 0.039**	*r* = 0.011, *p* = 0.921
	Latency to Bury	*r* = 0.155, *p* = 0.168	*r* = −0.137, *p* = 0.223
	Time Spent Burying	*r* = 0.068, *p* = 0.547	*r* = −0.111, *p* = 0.325
	Percent Buried	*r* = −0.069, *p* =	*r* = 0.077, *p* = 0.493
	Latency to	*r* = 0.049, *p* = 0.667	*r* = −0.082, *p* = 0.466
	Time Spent	*r* = −0.042, *p* =	*r* = 0.067, *p* = 0.552
	Immobile Episodes	*r* = −0.092, *p* =	*r* = 0.076, *p* = 0.501
Alcohol (*N* = 98)	Latency to enter	*r* = −0.107, *p* =	*r* = 0.025, *p* = 0.807
	Time in light	*r* = −0.133, *p* =	*r* = 0.116, *p* = 0.254
	Light Entries	***r* = 0.203, *p* = 0.045**	*r* = 0.018, *p* = 0.860
	Latency to Bury	***r* = −0.216, *p* =**	***r* =0.354, *p* < 0.001**
	Time Spent Burying	***r* = 0.397, *p*< 0.001**	***r* = −0.262, *p* = 0.009**
	Percent Buried	***r* = 0.216, *p* = 0.033**	*r* = −0.171, *p* = 0.093
	Latency to	*r* = −0.005, *p* =	*r* = −0.003, *p* = 0.974
	Time Spent	*r* = 0.031, *p* = 0.763	*r* = 0.077, *p* = 0.448
	Immobile Episodes	*r* = 0.084, *p* = 0.410	*r* = 0.059, *p* = 0.561

Table 2.1. Summary of the inter-relations between basal and stressor-induced increases in corticosterone (CORT) and our behavioral measures of negative affect, deconstructed along the between-subjects factor of Drinking. Sample sizes are indicated in parentheses. Significant correlations are indicated in bold.

2.3.6. Inter-Relations between Initial Alcohol Intake and Subsequent Alcohol Consumption

As clear sex- and age-related differences existed with respect to alcohol intake (**Figure 2.2.**), we conducted correlational analyses to determine whether or not group differences might exist with respect to the ability of initial alcohol intake to predict subsequent alcohol consumption. In adult mice, initial alcohol intake predicted their average total alcohol consumption (**Figure 2.14A**) (r (49) = 0.43, p = 0.002), but was inversely related to the extent to which alcohol intake escalated over the course of the 14-day drinking period (**Figure 2.14B**) (r (49) = −0.62, p < 0.0001). Initial alcohol intake also predicted the average alcohol consumption of adolescent mice (**Figure 2.14C**) (r (46) = 0.62, p < 0.0001), but did not reliably predict the extent of escalation (**Figure 2.14D**) (r (46) = −0.17, p = 0.27). Deconstructing the data along the Sex factor did not yield any significant, sex-specific, correlations that were distinct from those observed when both male and female subjects were combined (data not shown).

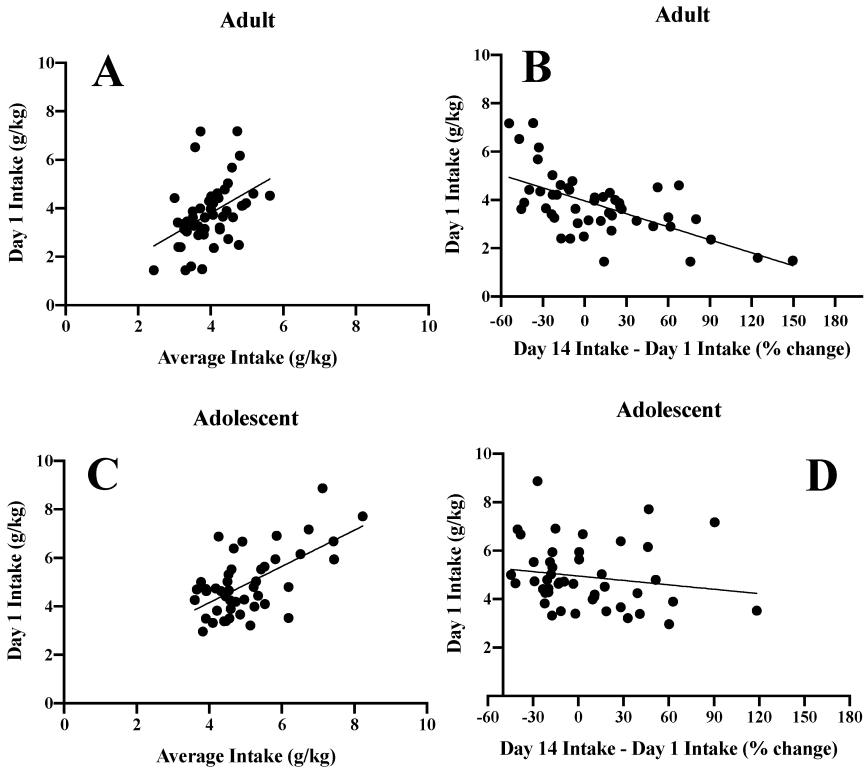


Figure 2.14. Summary of the significant inter-relations between measures of alcohol intake. A predictive relationship was observed between alcohol intake on Day 1 of drinking and the average alcohol intake over the 14-day drinking period in both (**A**) adult and (**C**) adolescent mice. Conversely, initial alcohol intake was inversely related to the escalation of intake observed over the course of the 14-day drinking period in both (**B**) adult and (**D**) adolescent mice.

2.3.7. Inter-Relations between Indices of Alcohol Intake and Behavioral Indices of Negative Affect

Given the failure to detect many alcohol-induced changes in negative affect using omnibus ANOVA approaches, we conducted correlational analyses to determine whether or not binge-drinking history might predict the magnitude of negative affect manifested during alcohol withdrawal as an alternative statistical approach to our dataset. As the marble-burying test yielded results most consistent with our prior reports, in addition to a positive relationship between CORT levels and behavior (**Table 2.1**), we conducted correlational

analyses between the behavioral measures from the marble-burying test and (1) the total alcohol consumption on the first day of binge-drinking, (2) the average total alcohol consumption across the 14 drinking days; and (3) the change in alcohol intake from Days 1 to 14 of alcohol-drinking (an index of drinking escalation). Curiously, when all binge-drinking mice were considered (N = 95–99), the average total alcohol intake was inversely correlated with the percent of marble buried ($r = -0.25$, $p = 0.01$), with no significant correlations detected regarding the relationship between initial alcohol intake and the escalation of alcohol intake and our three behavioral measures in this assay (r's < 0.149, p's > 0.20; data not shown).

Given the age- and sex- related differences in alcohol intake, the data were the deconstructed along these factors for re-analysis of their influence upon the relationship between our drinking measures and anxiety-like behavior in the marble-burying test. Consistent with the data from all mice mentioned above, initial alcohol intake did not predict any aspect of marble-burying behavior when the data was examined as a function of sex, age of drinking onset or withdrawal (data not shown). Curiously, when all female mice were considered, both the average alcohol intake, as well as the escalation of alcohol intake over the 14-day drinking period, predicted lower signs of anxiety-like behavior in the marble-burying test, as indicated by a positive relationship between the drinking measures and the latency to bury (**Table 2.2**) and an inverse relationship between the drinking measures and both the time spent burying (**Table 2.2**) and the percent of marble buried (**Figure 2.15A,B; Table 2.2**). Although the average alcohol intake did not predict subsequent marble-burying behavior in male mice (% buried in **Figure 2.15C**; see also **Table 2.2**), the extent to

which alcohol intake escalated during the binge-drinking phase of the study was positively correlated with both the time spent burying and the percentage of marbles buried (**Figure 2.15D; Table 2.2**). In contrast to the outcomes of the sex-related analysis, we failed to detect any major influence of the Age factor upon the relationships between alcohol intake and behavior in the marble-burying test (**Table 2.2**).

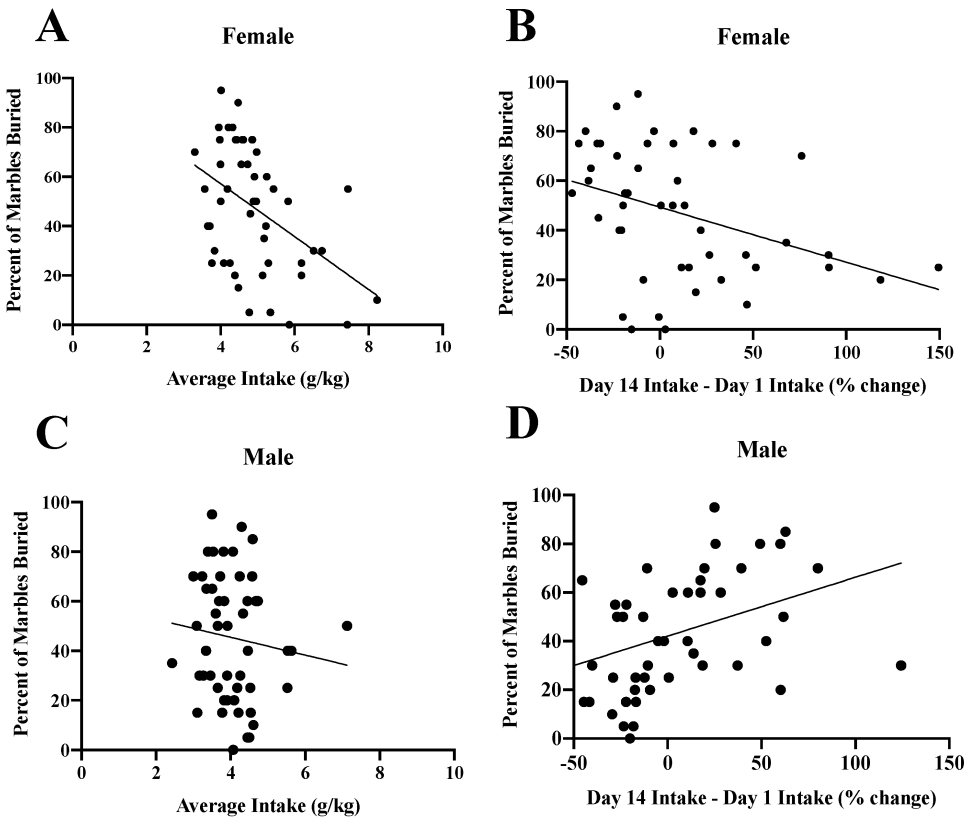


Figure 2.15. Summary of the inter-relations between the average alcohol intake (left), the escalation of alcohol intake (right) and the percent marble buried in the marble-burying test. In female mice, an inverse relationship was observed between both (**A**) alcohol intake and (**B**) the escalation of alcohol intake and the percent of marbles buried. (**C**) In male mice, no significant correlation was observed between the average alcohol intake and the percent of marbles buried. (**D**) However, an escalation of drinking predicted the percent of marbles buried in males.

Subject Factor	Measure	Latency to Bury	Time Burying	% Buried
Females (*N* = 49)	Ave. Intake	**$r = 0.313$, $p = 0.028$**	$r = -0.204$, $p = 0.160$	**$r = -0.442$, $p = 0.001$**
	Escalation	**$r = 0.327$, $p = 0.023$**	**$r = -0.309$, $p = 0.033$**	**$r = -0.373$, $p = 0.009$**
Males (*N* = 50)	Ave. Intake	$r = 0.224$, $p = 0.119$	$r = -0.146$, $p = 0.313$	$r = -0.113$, $p = 0.435$
	Escalation	$r = -0.136$, $p = 0.361$	**$r = 0.298$, $p = 0.042$**	**$r = 0.372$, $p = 0.010$**
Adolescents (*N* = 46)	Ave. Intake	$r = -0.117$, $p = 0.439$	$r = -0.141$, $p = 0.350$	$r = -0.145$, $p = 0.335$
	Escalation	$r = -0.123$, $p = 0.415$	$r = 0.135$, $p = 0.371$	$r = 0.150$, $p = 0.319$
Adults (*N* = 53)	Ave. Intake	$r = 0.260$, $p = 0.060$	$r = -0.023$, $p = 0.873$	$r = 0.015$, $p = 0.915$
	Escalation	$r = 0.271$, $p = 0.060$	$r = -0.106$, $p = 0.272$	$r = -0.215$, $p = 0.139$

Table 2.2 Summary of the inter-relations between the average alcohol intake and measures of anxiety-like behavior in the marble-burying test, expressed as a function of our between-subjects factors. the average alcohol intake and basal, as well as stressor-induced increases in, corticosterone (CORT) as a function of the independent variables investigated. The number of mice included in each individual analysis is indicated in parentheses. Significant correlations are indicated in bold. WD = withdrawal day.

2.4. Discussion

The goal of the present study was to directly interrogate sex differences in the age-related effects of binge-drinking upon negative affect expressed during early and protracted (70 days) withdrawal. We expected to replicate our prior observations from male mice indicating an interaction between the age of drinking-onset and withdrawal upon negative affect (Lee et al., 2015, 2016, 2017a-b, 2018a-c) and more recent findings suggesting a persistent increase in withdrawal-induced negative affect in female animals (Szumlinski et al., 2019). Based on evidence that female rodents tend to consume more alcohol than males (Finn et al., 2010; Moore et al., 2010; Strong et al., 2010; Melón et al., 2013; Cozzoli et al., 2014; Agabio et al., 2017; Becker et al., 2017; Logrip et al., 2018; Wellman et al., 2018; Wilsnack et al., 2018; Szumlinski et al., 2019), we hypothesized that female binge-drinking animals would exhibit more pronounced and/or enduring signs of withdrawal-induced negative affect than their male counterparts, irrespective of the age of drinking-onset. Using a 4-bottle-choice DID procedure, we replicated both age- (Moore et al., 2010; Melón et al., 2013; Szumlinski et al., 2019) and sex-related (Finn et al., 2010; Moore et al., 2010; Strong et al., 2010; Melón et al., 2013; Cozzoli et al., 2014; Agabio et al., 2017; Becker et al., 2017; Logrip et al., 2018; Wellman et al., 2018; Wilsnack et al., 2018; Szumlinski et al., 2019) differences in alcohol binge-drinking in mice, with adolescents consuming more alcohol than adults and females consuming more alcohol than males. The BACs for the adult-onset binge-drinking males were quite variable and their mean BAC on the day of sampling was just shy of the NIAAA 80 mg/dL criterion for binge-drinking (NIAAA, 2004). However, alcohol

intake resulted in BACs ≥ 80 mg/dL for the other groups tested, which is a finding in line with our prior studies using a 3-bottle-choice (10, 20, and 40% alcohol) DID drinking procedure (Lee et al., 2017b, 2018a-c; Szumlinski et al., 2019). Despite the sex difference in alcohol intake, we detected very few sex differences in the manifestation of withdrawal-induced negative affect. More concerning, when both sexes were tested concurrently, we failed to replicate not only age-dependent differences in withdrawal-induced negative affect in male mice but the direction of our alcohol effects tended to be opposite those reported in our prior studies of a single sex (Lee et al., 2015, 2016, 2017a-b, 2018a-c; Szumlinski et al., 2019).

Adult, male, mice with a 2-week history of binge-drinking under 3- or 4-bottle-choice DID procedures exhibit robust signs of anxiety-like behavior at one day withdrawal that dissipates by 30 days withdrawal (Lee et al., 2015, 2016, 2017a-b, 2018a-c). In contrast, male mice with a 2-week history of binge-drinking during adolescence are "resilient" to the negative affective state produced early in alcohol withdrawal, but a negative affective state incubates during alcohol withdrawal, manifesting robustly when the mice are adults (Lee et al., 2016, 2017a, 2018a-c). In our laboratory, this interaction between the age of binge-drinking-onset and alcohol withdrawal can be reliably detected in males, when mice are tested under light–dark shuttle-box, marble-burying, and forced swim procedures; other tests of anxiety-like behavior, such as the novel object encounter and elevated plus-maze, are less sensitive to the effects of alcohol withdrawal upon anxiety-like behavior, even for mice with a more extensive, 30-day, binge-drinking history (Lee et al., 2015). While the specific variables demonstrating alcohol-water differences in negative affect do vary from report to

report, the direction of the alcohol-water differences reported for both male (Lee et al., 2015, 2016, 2017a-b, 2018a-c) and female (Szumlinski et al., 2019) mice have been consistent with the interpretation that alcohol withdrawal induces a negative affective state. Moreover, in our hands, the light–dark shuttle-box, marble-burying and forced swim tests have also proven to be sensitive to age-related differences in basal anxiety-like behavior in alcohol-naïve mice, with the behavior of alcohol-naïve adolescents aligning with the interpretation of a hyper-anxious phenotype, particularly for males (Young et al., 2006; Lee et al., 2015, 2016, 2017a-b, 2018a-c). The reliability of our findings over the past several years is precisely the reason for selecting these behavioral paradigms for this large-scale study of the role for biological sex in mediating alcohol withdrawal-induced negative affect.

Indeed, some of the results of the present sex difference study do corroborate our earlier findings from studies employing a single sex. Male, adult-onset binge-drinking mice exhibited a longer latency to enter the light-side of shuttle-box on withdrawal Day 1—an effect no longer apparent at 70 days withdrawal (**Figure 2.3B**). In the marble-burying assay, female adolescent-onset, binge-drinking mice exhibited a shorter latency to begin marble-burying at one day withdrawal and this effect persisted for at least 70 days (**Figure 2.4A**). In contrast, male adolescent-onset, binge-drinking mice exhibited a shorter latency to begin burying only at the 70-day withdrawal time-point (**Figure 2.4A**)—a finding consistent with an incubation of a negative affective state in male adolescent drinkers (Lee et al., 2017a) and the first demonstration by our group that incubated affective state persists beyond 30 days withdrawal. Additionally, both male and female adult-onset, binge-drinking mice exhibited a shorter latency to begin marble-burying on withdrawal day 1, relative to their water-drinking

controls (**Figure 2.4B**). However, in contrast to prior reports (Lee et al., 2016), this effect was still apparent at 70 days withdrawal (**Figure 2.4B).** Although no significant sex difference was detected by omnibus ANOVA, the enduring nature of the effect of adult-onset binge-drinking upon the latency to marble-bury aligns with that reported previously for binge-drinking, female mice (Szumlinski et al., 2019) and may be driven largely by the female subjects.

To the best of our knowledge, only one other published study has attempted to examine directly for sex differences in interactions between the age of binge-drinking onset under DID procedures, alcohol withdrawal and negative affect (Szumlinski et al., 2019). In this earlier study from our group, all of the male and female mice exhibited BACs considerably lower than typically observed under DID procedures (~30 to 70 mg/dL)—a finding we eventually attributed to an insufficient period of acclimation to the drinking cages prior to alcohol presentation (Szumlinski et al., 2019). Not surprisingly given the low BACs of the mice, we detected no water–alcohol differences in negative affect in this earlier study, precluding any conclusions regarding sex differences or subject factor interactions in our affective measures (Szumlinski et al., 2019). In contrast to our earlier report (Szumlinski et al., 2019), the BACs detected herein were near to, or above, the 80 mg/dL NIAAA criterion for binge-drinking (**Figure 2.2F**). Despite this, the vast majority of the affective measures in the present study either failed to indicate water-alcohol differences (e.g., marble-burying; **Figure 2.4**) or indicated a counter-intuitive result whereby water controls exhibited more anxiety-like behavior than their alcohol-experienced counterparts. While the interpretation of the direction of alcohol's effects upon behavior in the forced swim test is

controversial (see Ref. (Lee, et al., 2017b) for discussion), we were particularly struck by relatively high levels of anxiety-like behavior exhibited by water controls in the light–dark shuttle-box test and the polar opposite water-alcohol differences observed for both adult and adolescent mice in this paradigm (**Figure 2.3**) versus those reported by our group previously in studies of either male (Lee et al., 2015, 2017a, 2018a-c) or female (Szumlinski et al., 2019) mice. While it is true that marked procedural differences existed with respect to the daily handling and housing of water- versus alcohol-drinking animals in the present study, the procedures employed herein where nearly identical to those employed in our published work over the past 3 years in which very clear alcohol-water differences in affective behavior were detected in both male (Lee et al., 2017a, 2018a-c) and female mice (Szumlinski et al., 2019). In fact, the first several cohorts of this sex difference study were conducted in parallel with some of the later "female only" cohorts summarized in Szumlinski et al. (2019), which successfully replicated many of our reported effects of alcohol withdrawal upon affective behavior in female subjects (Szumlinski et al., 2019).

We were also struck by the very few age-related differences in basal anxiety-like behavior exhibited by water-drinking controls during early withdrawal in the present study as our prior work reliably detected higher behavioral indices of anxiety in adolescent versus adult males (Lee et al., 2015, 2016, 2017a-b, 2018a-c) and females (Szumlinski et al., 2019). Herein, water-drinking adolescents exhibited the shortest latency to begin floating (**Figure 2.9**), as well as most floating behavior (**Figure 2.10** and **Figure 2.11**), when assayed on WD1—findings indicative of age-related differences in the basal affective response to, or coping strategy employed in, the forced swim test. Curiously, this is the first time we have

detected adolescent-adult differences in the floating behavior manifested by water-drinking controls on WD1; in our earlier reports, the amount of floating/swimming, as well as the latency to first float, were both comparable between water-drinking adolescent and adult mice tested on WD1, although marked differences in anxiety-like behavior were detected in the light–dark box and marble-burying tests (Lee et al., 2016; Szumlinski et al., 2019). Aligning with our published studies in mice (Lee et al., 2015, 2016, 2017a-b, 2018a-c; Szumlinski et al., 2019), age-related differences in affective measures have been consistently reported in drug/alcohol-naïve rats, as has a resiliency to the negative affective state produced by early alcohol withdrawal in adolescent animals (Spear, 2000, 2002; Varlinskaya & Spear, 2004). Thus, both the relative lack of adolescent-adult differences in baseline emotionality (particularly in the light–dark box and marble-burying tests) and in the responsiveness to early alcohol withdrawal were very unexpected. At the present time, it is unclear why our adolescent animals behaved so differently from the mice in our prior work. This being said, one major procedural difference between this and prior work (at least from our laboratory) relates to the concurrent testing of male and female subjects. In partial support of this, chemosensory social stimuli, such as those in vaginal secretions, differentially alter neuronal activity within the mesocorticolimbic system of adolescent versus adult males to affect motivated behavior (Romeo et al., 1998; Bell et al., 2013a-b)—an effect attributed to the differential maturation state of the brain, rather than changes in circulating testosterone (Bell et al., 2013a-b). While this line of chemosensory research has focused on measures of conditioned reward, it is entirely possible that exposure to pheromones from adults of the opposite sex during anxiety testing may have unpredictably impacted the behavior of the adolescent mice in the present study.

Related to this, concurrent testing of male and female subjects may have also mitigated the negative affective state produced by a history of binge-drinking in adults. In support of this notion, exposure to adult female urinary pheromones during elevated plus-maze testing produces a testosterone-driven anxiolytic effect, without impacting locomotor activity in this assay (Aikey et al., 2002). Such a finding aligns with other research indicating an anxiolytic effect of circulating testosterone in adult male laboratory mice and rats, to include behavior in the marble-burying test (Fernandez-Guasti & Picazo, 1992; Frye et al., 2008). Thus, the blunted negative affective state produced by alcohol withdrawal exhibited by the male mice herein could very well reflect a testosterone-dependent anxiolysis, elicited by the presence of female pheromones during testing. While it is known that emotionality varies with the estrous cycle in adult female rodents (Fernandez-Guasti & Picazo, 1992), to the best of our knowledge, it remains to be determined whether exposure to adult male pheromones elicits a comparable anxiolytic effect in either adolescent or adult female subjects to account for their blunted negative affective state observed herein. Future work seeks to better understand how exposure to urinary pheromones from the opposite sex impacts anxiety-related measures in both adult and adolescent mice to alter the expression of such measures during alcohol withdrawal.

Chapter 3:

Modulation of marble-burying behavior in adult versus adolescent C57BL/6J mice by ethologically relevant chemosensory stimuli

3.1 Introduction

Affective disorders exhibit a very high degree of comorbidity with alcohol use disorders (SAMHSA, 2010), with the global prevalence of an affective disorder diagnosis being twice as high in women compared to men (UNODC, 2023). Concerningly, women advance through the addiction landscape at a more rapid rate than men (UNODC, 2023; Wilsnack et al., 2018; Agabio et al., 2017; Becker et al., 2017) and exhibit higher rates of comorbid psychiatric conditions, including affective disorders (Grant et al., 2004; Hasin et al., 2007; Johnston et al., 2008; Kessler et al., 2005; Merikangas et al., 2007). The sexual dimorphism in alcohol use disorders, affective disorders, and their comorbidity is theorized to appear early in adolescence (Johnston et al., 2008; Fox & Sinha, 2009; Sonne et al., 2003; Witt, 2007); however, it is difficult to disentangle cause-effect relationships through studies of humans in an experimentally controlled fashion. Thus, we (Jimenez Chavez et al., 2020, 2022, 2023; Szumlinski et al., 2023) and others, e.g., (Logrip et al., 2018; Strong et al., 2010; Cozzoli et al., 2014; Melón et al., 2013), have employed animal models to try to understand how biological sex interacts with the age of alcohol-drinking onset to impact brain and behavior. However, despite assaying for behavioral signs across a number of different paradigms, our recent large-scale efforts to identify sex differences in the age-selective effects of alcohol withdrawal on negative affect (Jimenez Chavez et al., 2022, 2023) have failed to replicate our prior results derived from studies of a single-sex (Lee et al., 2015, 2016, 2017, 2018a-c), with either no or inverse age-related differences observed for both basal and alcohol withdrawal-induced anxiety-like behavior. These failures to replicate our prior work challenged us to determine what procedural variables might have negatively impacted our ability to detect not only our basic finding that early alcohol withdrawal

induces a negative affective state in adult mice (Lee et al., 2015, 2016, 2017, 2018a-c), but age differences therein (Lee et al., 2016, 2017, 2018a-c).

In this report, we examined the possibility that sex-related pheromones might modulate affective behavior expressed in one of the assays that reliably detected age differences in alcohol withdrawal-induced negative affect when mice of a single sex were examined (Lee et al., 2015, 2016, 2017, 2018a-c) – the marble-burying test. In this paradigm, a shorter latency to begin marble-burying and a higher frequency of this behavior is generally interpreted as increased levels of anxiety-like behavior or heightened negative affect. Although some have questioned the specificity of marble-burying as an indicator of anxiety, c.f., (Thomas et al., 2009), the marble-burying assay has high bidirectional, predictive, validity for anxiety-like behavior as burying is reduced by pretreatment with various anxiolytic (Broekkamp et al., 1986; Njung'e & Handley, 1991a; Ichimaru et al., 1995; Borsini et al., 2002; Nicolas et al., 2006) or antidepressant drugs (Broekkamp et al., 1986; Njung'e & Handley, 1991a, 1991b; Takeuchi et al., 2002; de Brouwer et al., 2019) and increased by stimulation of the central noradrenergic system (den Hartog et al., 2020; Lustberg et al., 2000; Anton & Becker, 1995). Similarly, in our hands, unpleasant or aversive psychophysiological conditions in humans, such as early alcohol withdrawal (Anton & Becker, 1995; Roelofs, 1985), reliably augment marble-burying behavior in adult male C57BL/6J (**B6**) mice (Lee et al., 2015, 2016, 2017, 2018a-c), with a similar effect reported for B6 females (Lee et al., 2018c). In contrast, but consistent with the human condition c.f. (Spear & Varlinskaya, 2005), adolescent B6 mice are resilient to this anxiogenic/dysphoric state in early alcohol withdrawal, but manifest increased marble-burying behavior later in

adulthood e.g., (Lee et al., 2016, 2017, 2018a-c). As exposure to female-related pheromones are reported to lower fear responses and induce greater risk taking in adult male mice (Kavaliers et al., 2001, 2008, 2012), we hypothesized that our failure to replicate age-related differences in both basal and alcohol withdrawal-induced negative affect, particularly in male mice, might relate to the inadvertent exposure to female pheromones during testing. Further, as the behavioral response to sex-related pheromones can vary depending on the animal's biological sex, reproductive physiology and experience (Kavaliers et al., 2001; Stowers & Liberles, 2016; Stowers & Kuo, 2015), the possibility existed that exposure to odors of the opposite sex may have also impacted our ability to detect age-related differences in marble-burying during alcohol withdrawal when both sexes were examined concurrently.

To the best of our knowledge, no study has explicitly examined how exposure to odors of the same or opposite sex impacts behavior in the marble-burying test, let alone how effects might vary as a function of biological sex or sexual maturity. Thus, the present study was designed to examine how sex-related pheromones alter marble-burying behavior in sex- and drug-naive adult and adolescent mice of both sexes. To facilitate interpretation of our results, follow-up studies examined for sex by age interactions in the effects of a novel neutral odor (vanilla) (Yang & Crawley, 2009; Wersinger et al., 2007) versus a novel, aversive odor (tea tree) (Quadir et al., 2019) on marble-burying. Our results replicate the anxiogenic effect of tea tree odor on marble-burying (Quadir et al., 2019), but also provide new evidence for clear sex- and age-dependent effects of exposure to male and female pheromones, as well as a neutral vanilla odor, on marble-burying behavior. These findings are of significant relevance to the experimental design and procedural timing of experiments

including the subject factors of sex and age, particularly when comparing between adult and adolescent subjects.

3.2. Materials and Method

3.2.1. Subjects

The subjects of this study included male and female adult (PND 56+) and adolescent (PND 28-29) C57BL/6J mice (see Experimental Designs for more details). All mice were housed in same-sex, age-matched, groups of 2-4 per cage that were situated on ventilated racks. These polycarbonate cages contained sawdust bedding, nesting materials and an enrichment device and were located in a colony room with regulated climate and humidity. All mice were acclimated to a 12-hour reversed light cycle, with lights turning off at 11:00 AM, in accordance with vivarium guidelines. To ensure minimal stress during testing, a minimum 7-day acclimation period was implemented, during which mice experienced limited handling restricted to tail-marking procedures. All experimental procedures were in compliance with The Guide for the Care and Use of Laboratory Animals (National Research Council, 2011) and approved by the Institutional Animal Care and Use Committee of the University of California, Santa Barbara.

3.2.2. Marble-burying Procedures.

In preparation for behavioral testing, mice were relocated from the colony room to a designated testing room within the vivarium, where they remained in their home cages for a 30-minute acclimation period prior to testing. For all experiments, a sex- and age-specific testing schedule was implemented to nullify the influence of subject-related odors from the

opposite sex or differentially aged mice (i.e. odors not associated with the different bedding conditions employed). Mice were then subjected to the marble-burying assay over a span of two or three consecutive days, during which they encountered a different bedding condition each day. With some exceptions, detailed in the Experimental Designs section below, the sequence of bedding conditions was counterbalanced to mitigate order effects.

For testing, each mouse was introduced to a polycarbonate testing box (12 cm × 8 cm × 6 cm), which contained 5 cm of sawdust bedding (P.J Murphy Forest Products, Montville, NJ, USA) from one of the different bedding conditions (see Bedding Collection/Preparation below) and 20 uniformly distributed, round black glass marbles, arranged in a 4x5 grid. For a 20-minute testing period, mice were free to explore and bury marbles where a more burying behavior (assayed by number of marbles buried at the end of the session and/or total time spent burying) greater frequency of burying behavior and a shorter latency to begin burying marbles served as indices of heightened negative affect. After each testing session, the total number of marbles covered 2/3rds by the bedding was documented, and the mice were returned to their home cages. Test boxes were completely emptied of bedding, sprayed thoroughly with a virucidal spray (Rescue^{TM}; Virox Technologies Inc., Oakville, ON, Canada) and then wiped dry, prior to the addition of new bedding for the next animal. Upon the completion of testing at the end of the day, all testing cages were sent for washing and cleaned cages were employed the next day. For Experiments 1 and 2, behavior was digitally video-recorded using AnyMaze tracking software (Stoeling Co., Wood Dale, IL, USA) for playback and determination of distance traveled (in m). The latency to start burying and/or the total time spent burying were then recorded by a blind observer using a stopwatch upon

video playback, as conducted in previous studies, e.g., (Jimenez Chavez et al., 2020, 2023). For Experiment 3, a computer malfunction required that we score behavior manually as conducted in a recent study by our group (Jimenez Chavez et al., 2022) and thus, the data for the total time spent burying and the distance traveled could not be determined for this study.

3.2.3. Bedding Collection/Preparation.

For Experiment 1, we collected bedding that had been soiled over a six-day period from the home cages of adult, sex- and drug-naïve, B6 female and male mice 24 hours prior to testing for marble-burying. The soiled bedding was always derived from cages of unfamiliar, adult, mice. To preserve the distinct scent profiles, the soiled bedding was collected and stored separately in sealed containers for each sex. On the test day, a 5 cm bedding mix was used, consisting of equal parts of soiled and unscented bedding for each mouse. For Experiments 2 and 3, the scented bedding (vanilla or tea tree) was freshly prepared on the day of testing. Clean, unscented, bedding was infused with artificial vanilla extract (McCormick & Co., Inc., Hunt Valley, MD, USA) or tea tree oil (rareESSENCE, LLC; Minneapolis, MN, USA) by sprinkling 5-6 drops onto the bedding in each apparatus and then thoroughly mixed to ensure uniform distribution of scent. To augment the sensory environment, an approximately 5 cm stripe of the vanilla extract or the tea tree oil was applied around the interior of the polycarbonate cage with a moistened Kimwipe (Kimberly-Clark, Irving, TX, USA), as conducted in prior studies of predator odor exposure conducted by our group (Wiedenmayer, 2004; Keyes et al., 2008).

3.2.4 Experimental Designs

The primary objective of this study was to examine how ethologically relevant chemosensory stimuli within the marble-burying test modulate the burying behavior of both adolescent and adult, male and female, sex-naïve B6 mice. This study included three distinct, yet interrelated, experiments as detailed below. The experimental designs of the three experiments are graphically depicted in **Figure 1.**

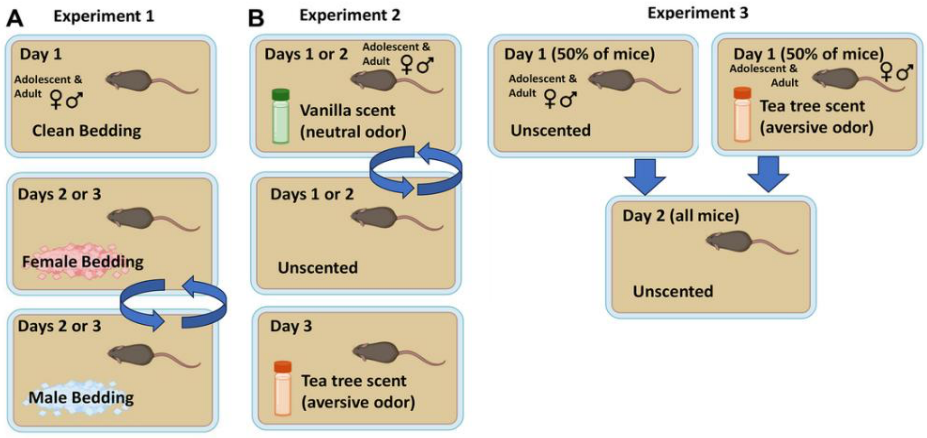


Figure 3.1. Summary of the experimental designs and procedural timelines of the three experiments described in this report. All images were created in BioRender (biorender.com).

Experiment 1: The first experiment was designed to assay the influence of socially relevant chemosensory stimuli (odors from male and female mice) on three indices of affect in the marble-burying test: latency to first bury to gauge initial behavioral responsiveness to the marbles, as well as the time spent burying and total number of marbles buried as indices of negative affect, e.g., (Njung'e & Handley, 1991b). As the perception of odors from the opposite sex change during sexual development, e.g., (Stowers & Liberles, 2016), this study compared the marble-burying behavior of male and female adolescent, as well as male and female adult, mice in the presence of unscented bedding (i.e., standard procedures; control condition; Day 1 for all mice), or unscented bedding mixed with soiled bedding from cages

of unfamiliar adult male or female mice (respectively, male- and female-soiled; see **Figure 3.1A**). The order of testing under the soiled bedding conditions was counterbalanced across the next two consecutive days within each age and sex. To minimize the influence of sex-related pheromones of the subjects, males and females were tested on distinct days and adolescents were tested separately from adults. Experiment 1 employed 48 mice (n=12/sex/age) and all mice were obtained from The Jackson Laboratory (Sacramento, CA) and allowed to acclimate to the reverse cycle for at least 1 week prior to behavioral testing.

Experiment 2: Experiment 2 was designed to facilitate the interpretation of the effects of inherently motivating sex-specific chemosensory stimuli on marble burying behavior (i.e., do increased indices of marble-burying in the presence of a sex-related odor reflect a response to an aversive/anxiogenic stimulus or a mere response to a novel odor?). Specifically, we compared the marble-burying behavior of male and female mice, both adolescent and adult, in the presence of a novel but neutral, odor (artificial vanilla extract) (Yang & Crawley, 2009; Wersinger et al., 2007) against a novel, but inherently aversive, odor (tea tree oil) (Quadir et al., 2019). Experiment 2 also employed a within-subjects design in which mice were exposed to all three bedding conditions. However, out of concern that the tea tree odor might induce a conditioned place-aversion that could impact behavior in the presence of the unscented and vanilla-scented conditions (Wiedenmayer, 2004), we counterbalanced the presentation of unscented and vanilla odor over the first two days of testing and then all mice were tested in the presence of the tea tree odor on the third and final day (see **Figure 3.1B**). As in Experiment 1, a total of 48 mice were employed in Experiment 2 (n=12/sex/age). However, due to pandemic-related difficulties acquiring adult mice, the adult mice in this

study were obtained from our breeding colony at UCSB (breeders obtained originally from The Jackson Laboratory; offspring tested PNDs 60-75), while the adolescent mice were obtained directly from The Jackson Laboratory as in Experiment 1.

Experiment 3: The results of Experiment 2 confirmed that tea tree odor increased marble-burying behavior. Thus, we deemed it important for future work to determine whether initial exposure to tea tree oil would induce a conditioned aversive response to the marble-burying apparatus that might impact subsequent marble responsiveness. Although published studies indicated that marble-burying behavior under standard testing conditions neither habituates nor sensitizes with repeated testing in mice, e.g., (Thomas et al., 2009), we first conducted a pilot study in adult male B6 mice from our breeding colony (aged 6-9 months) in which mice were tested in the presence of unscented bedding over the course of two consecutive days. Having established that the marble-burying behavior of mice from our breeding colony is also stable across days, we examined for carry-over effects of the tea tree odor in male and female, adolescent (PND 27-28) and adult mice (PND 60-75). Unfortunately, a computer malfunction around the time of testing precluded video-recording of behavior for playback to determine the latency to bury, the total time spent burying and the total distance traveled. Thus, only the data for the number of marbles buried is reported for Experiment 3. Consistent with Experiment 2, the adult mice were obtained from our breeding colony and the adolescent mice were obtained from The Jackson Laboratory. Experiment 3 employed a mixed design in which half of the mice were exposed to the unscented bedding across two consecutive days, while the other half of the mice were initially exposed to tea tree-scented

bedding, followed by unscented bedding to examine for carry-over effects (see **Figure 3.1C**). This study employed n=7 adult mice/sex and n=8 adolescent mice/sex.

3.2.5. Statistical Analyses and Graphical Depiction of Findings.

The data were analyzed using mixed-model ANOVAs, with Age and Sex as between-subjects variables and Bedding as the repeated measure variable for all analyses. Both the F statistics and effects sizes are reported. Significant main effects in the absence of any significant interaction(s) were followed by LSD post-hoc tests. Significant interactions were deconstructed along the relevant factors, followed by tests for simple effects with LSD corrections (when >2 comparisons were required) or paired samples t-tests (when 2 comparisons were required). Although the LSD method of post-hoc analyses is less conservative than other corrections (e.g., Tukey's tests), it offers greater statistical power to detect group differences, which aligns with the exploratory nature of our study to discover potential factors that may modulate behavior. For all analyses, alpha was set at 0.05. When the assumption of sphericity was not met, analyses were corrected using the Greenhouse–Geisser adjustment. Extreme outliers, defined by ± 3 × IQR, were removed. With this exclusion criterion, 1 adolescent female mouse was removed from the analysis of the latency to bury from Experiment 1, 1 adolescent female, 2 adolescent males and 1 adult female were removed from the analysis of the latency to bury from Experiment 2. No other animals were noted as extreme outliers. IBM SPSS Statistics software (version 27.0 for Macintosh) was used for all statistical tests. For transparency, the results for adult male, adult female, adolescent male and adolescent female mice across the specific bedding conditions employed in each experiment are always presented, regardless of the statistical outcome for the specific

analysis. Graphs summarizing significant interactions between our factors are also provided to facilitate visualization of the statistical results. When no interactions are observed between factors, significant main effects are also provided. GraphPad Prism software (version 10 for Macintosh) was used to create all graphs.

3.3. Results

3.3.1. Experiment 1: Sex by Age interactions in the effects of social chemosensory stimuli on marble-burying behavior

To test the hypothesis that the apparent anxiolytic effect of female odors reported in prior studies of adult male mice (Kavaliers et al., 2001, 2008, 2012) extends to marble-burying procedures, we employed a within-subjects design in which we compared marble-burying behavior in the presence of unscented bedding on day 1 of testing, followed by exposure to female- or male-soiled bedding in a counter-balanced fashion across days 2 and 3 of testing (see **Figure 3.1A**). To examine whether the effects of social chemosensory stimuli were specific to adult males, the study also included adult females, as well as both female and male adolescent mice. Based on the limited literature, e.g., (Stowers & Liberles, 2016), it was predicted that adolescent mice of both sexes, as well as adult females, would exhibit anxiety-like behavior as manifested by increased marble-burying in the presence of the bedding of adult males.

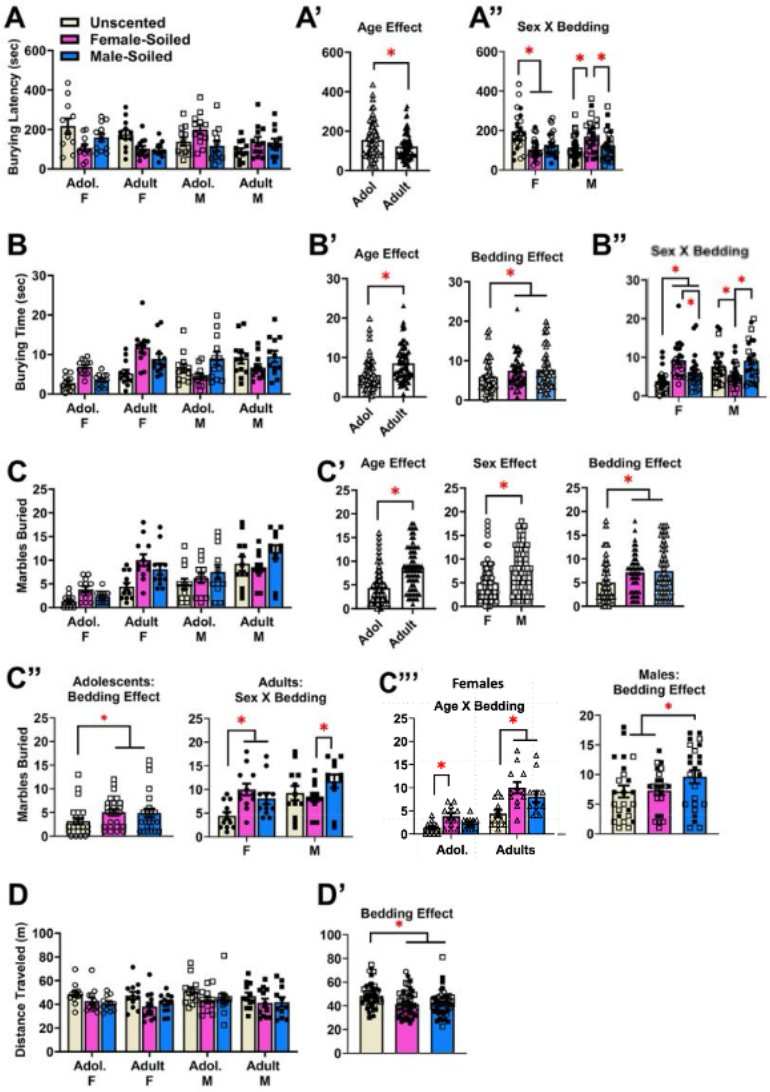


Figure 3.2. Summary of the effects of social chemosensory stimuli on the marble-burying behavior of adolescent and adult mice (Experiment 1). (**A**) Summary of the latency to first begin marble-burying exhibited by the adolescent (Adol.) and adult male (M) and female (F) mice in the presence of unscented, female- and male-soiled bedding for which a significant Sex by Bedding Condition interaction was detected [females: Adol. unscented (n = 11), female-soiled (n = 11), male-soiled (n = 12); for all other groups (n's = 12)]. **(A')** Depiction of the significant Age effect for the latency to bury. **(A")** Depiction of the significant Sex x Bedding interaction for the latency to bury. **(B)** Results for the total time spent burying marbles indicated a significant Sex by Bedding Condition interaction [all group n's = 12]. **(B')** Depiction of the significant main effects of Age and Bedding Condition for the time spent burying. **(B")** Depiction of the significant Sex x Bedding Condition interaction for the time spent burying. **(C)** Summary of the number of number of marbles buried by the mice in Experiment 1. **(C')** Depiction of the significant Age, Sex and Bedding Condition effects for the number of marbles buried. **(C")** Deconstruction of the significant 3-way interaction along the Age factor indicated a significant main Bedding

Condition effect for adolescent mice and a significant Sex x Bedding Condition interaction for adults. **(C''')** Deconstruction of the significant 3-way interaction along the Sex factor revealed a significant Age x Bedding interaction for female mice and a significant Bedding Condition effect for males. **(D)** Summary of distance traveled (in m) during Experiment 1 [adult males: unscented (n = 12), female-soiled (n = 12), male-soiled (n = 11); all other group n's = 12]. **(D')** Depiction of the significant main Bedding Condition effect for the distance traveled. The bar graphs represent the means ± SEMs of the number of individual mice indicated above. *$p < 0.05$ for indicated comparisons (LSD post-hoc tests).

3.3.1.1. Latency to bury: A summary of the group differences in the latency to bury by the mice in Experiment 1 are presented in **Figure 3.2A.** A mixed-model ANOVA was conducted to investigate the effect of Age (adolescent vs. adult), Sex (female vs. male), and Bedding Condition (unscented, female-soiled, and male-soiled) on the latency to first exhibit marble burying. The analysis yielded a significant main effect of Age (Adolescents > Adults; **Figure 3.2A'**) [F(1,43) = 5.58, p = 0.023, eta = 0.115]. In contrast, the main effects of Sex [F(1,43) = 0.19, p = 0.664, eta = 0.004] and Bedding Condition [F(2,86) = 2.22, p = 0.115, eta = 0.049] were not statistically significant.

Interaction effects were also examined and although the ANOVA failed to detect a significant three-way interaction [3-way interaction: F(2,86) = 2.74, p = 0.070, eta = 0.060], a significant two-way interaction between Sex x Bedding Condition was found [F(2,86) = 12.98, p <.001, eta = 0.232]. Thus, the data were collapsed across Age and analyzed separately for male and female mice to determine how the soiled bedding influenced their initial response to the marbles. As depicted in **Figure 3.2A",** the latency of females to bury marbles was significantly shorter in female-soiled (p <.001) and male-soiled bedding (p = 0.002) compared to the unscented bedding condition, with no significant differences observed between the female- and male-soiled bedding (p = 0.269). As also illustrated in **Figure 3.2A"** and in contrast to females, male mice exhibited a longer latency to bury when

tested in female-soiled bedding, relative to both the unscented ($p = 0.011$) and male-soiled conditions ($p = 0.026$), with no effect of male-soiled bedding detected ($p = 0.643$). Thus, irrespective of age, these data for the latency to first marble bury indicate that female odors exert an anxiolytic effect in male mice, social chemosensory stimuli from both sexes are anxiogenic in females.

3.3.1.2. Total time spent burying. A summary of the group differences in the total time spent burying by the mice in Experiment 1 are presented in **Figure 3.2B.** An Age x Sex x Bedding Condition mixed-model ANOVA was employed to examine for group differences in the total time spent marble burying. Results indicated significant main effects of Age (Adults > Adolescents; **Figure 3.2B', left**) [$F(1,44) = 11.05$, $p = 0.002$, eta = 0.201] and Bedding Condition (Female-soiled vs. Unscented, $p = 0.007$; Male-soiled vs Unscented, $p = 0.002$; Female-soiled vs. Male-soiled, $p = 0.574$; **Figure 3.2B', right**) [$F(2,88) = 6.95$, $p = 0.002$, eta = 0.136], but not of Sex [$F(1,44) = 1.35$, $p = 0.252$, eta = 0.030]. While the 3-way interaction was not statistically significant [3-way interaction: $F(2,88) = 0.56$, $p = 0.572$, eta = 0.013], a significant Sex x Bedding Condition was observed [$F(2,88) = 30.98$, $p < 0.001$, eta = 0.413] that is depicted in **Figure 3.2B"**. As illustrated, simple main effects analysis with LSD post-hoc corrections revealed that female mice spent a longer time burying marbles in both the female-soiled ($p < 0.001$) and male-soiled bedding ($p = 0.007$), compared to the unscented bedding condition**.** Moreover, females spent more time marble burying in the female- versus male-soiled bedding ($p < 0.001$). In contrast, male mice spent less time burying marbles in the female-soiled bedding than in the unscented ($p = 0.007$) and male-soiled bedding ($p < 0.001$)**,** with no significant difference detected between the unscented and

male-soiled bedding conditions (**Figure 3.2B"**; p = 0.062). These results further the notion that female odors exert an anxiolytic effect in male mice, while both male and female odors are anxiogenic in female mice.

3.3.1.3. Number of marbles buried. A summary of the group differences in the number of marbles buried by the mice in Experiment 1 are presented in **Figure 3.2C.** We also conducted an Age x Sex x Bedding Condition mixed-model ANOVA to evaluate the total number of marbles buried during each testing session. Significant main effects were observed for Age (Adults > Adolescents; **Figure 3.2C', left**) [F(1,44) = 21.91, p <.001, eta = 0.332], Sex (Males > Females; **Figure 3.2C', middle**) [F(1,44) = 10.95, p = 0.002, eta = 0.199] and for Bedding Condition (Male- and Female-Soiled > Neutral, p's<0.001; **Figure 3.2C', right**) [F(2,88) = 13.49, p <.001, eta = 0.235].

Furthermore, a significant three-way interaction (Age x Sex x Bedding Condition) was detected [F(2,88) = 3.36, p = 0.039, eta = 0.071], indicating that the effects of Bedding Condition on marble-burying behavior varied as a function of both Age and Sex. To investigate further, the ANOVA was first deconstructed along the Age factor to examine the Sex by Bedding Condition interaction separately for adolescents and adults. For the adolescent mice, the Sex x Bedding interaction was not significant [F(2,44) = 2.58, p = 0.087, eta = 0.105], however, a main effect of Bedding Condition was detected for adolescent mice [F(2,44) = 5.99, p = 0.005, eta = 0.214], which is depicted in **Figure 3.2C" (left)**. As illustrated, LSD post-hoc tests indicated that adolescent mice buried more marbles in both the female-soiled (p = 0.004) and male-soiled bedding (p = 0.003), compared to the unscented

bedding, with no significant differences between the male-versus female- soiled bedding conditions ($p = 0.860$). For the adults, a significant Sex x Bedding Condition interaction was observed [$F(2,44) = 9.13$, $p < 0.001$, eta = 0.293], which is depicted in **Figure 3.2C" (right)**. As illustrated, test for simple main effects with LSD corrections indicated that adult female mice buried more marbles in the presence of the female-soiled ($p < 0.001$) or male-soiled bedding conditions ($p = 0.008$), relative to unscented bedding, with no difference detected between the female-soiled and male-soiled conditions ($p = 0.094$). In contrast, adult male mice buried more marbles in male-soiled bedding compared to female-soiled bedding ($p = 0.005$); however, the number of marbles buried did not differ significantly between the unscented condition and the female- soiled ($p = 0.402$) or male-soiled bedding conditions ($p = 0.061$).

The significant 3-way ANOVA was then deconstructed along the Sex factor to explore age-related differences within each bedding environment within each sex. For females, the Age x Bedding Condition interaction was significant [$F(2,44) = 5.08$, $p = 0.010$, eta = 0.188]. As illustrated in **Figure 3.2C"' (left)**, subsequent analysis for simple main effects with LSD post-hoc corrections revealed that adolescent females buried more marbles in the presence of female-soiled bedding than unscented bedding ($p = 0.001$), with no other significant group differences detected (unscented vs. male-soiled, $p = 0.160$; female vs. male-soiled; $p = 0.094$). In contrast, adult females buried more marbles in both the female -soiled ($p < 0.001$) and male-soiled environments ($p < 0.001$), compared to the unscented bedding. Moreover, female buried more marbles in the female-vs. the male-soiled bedding ($p = 0.036$; see **Figure 3.2C"', left**). For males, the Age x Bedding ANOVA failed to indicate a

significant 2-way interaction [F(2,44) = 1.05, p = 0.359, eta = 0.045], but a significant overall main effect of Bedding Condition was observed that is illustrated [F(2,44) = 5.22, p = 0.009, eta = 0.192]. As shown in **Figure 3.2C"' (right)**, LSD post-hoc tests indicated that this main effect reflected more marble burying in the male-soiled bedding, than both the female-soiled (p = 0.012) and unscented bedding conditions (p = 0.013), with no significant difference in the number of marbles buried between the unscented vs. female-soiled bedding environments (p = 0.824). These data for the number of marbles buried suggest that this variable may be most sensitive to age-related differences in the effects of socially relevant odors on behavior in this assay, revealing an anxiogenic effect of both male- and female odors.

3.3.1.4. Distance Traveled. Analysis of the distance traveled by the mice during the 20-minute marble-burying session (**Figure 3.2D**) did not detect significant main effects for Age [F(1,43) = 1.08, p = 0.305, eta = 0.024] or Sex [F(1,43) = 0.43, p = 0.783, eta = 0.010]. However, a significant main effect of Bedding Condition was observed (Unscented > Female- and Male-soiled; p's<0.001) that is depicted in **Figure 3.2D'** [F(2,86) = 12.98, p < .001, eta = 0.232]. The analysis also revealed comparable locomotor activity between the female-soiled and male-soiled bedding conditions was comparable (p = 0.620). This analysis detected no significant Age x Sex x Bedding Condition interaction [F(2,86) = 0.22, p = 0.802, eta = 0.005], no significant Sex x Bedding Condition [F(2,86) = 0.07, p = 0.931, eta = 0.002] or Age x Bedding Condition interactions [F(2,86) = 0.45, p = 0.638, eta = 0.010]. These data for the distance travelled indicates that the presence of male or female odors lowers locomotor activity, regardless of the age or sex of the mice tested. Thus, an inverse relationship exists between the effects of socially relevant odors on psychomotor activation

and our indices of anxiety-like behaviors in this assay, which may reflect the fact that marble-burying is physically incompatible with forward locomotion (i.e., animal is stationary over a marble when burying).

3.3.2. Experiment 2: Sex by Age interactions in the effects of neutral versus aversive chemosensory stimuli on marble-burying behavior

To facilitate the interpretation of the results from Experiment 1, we conducted a second experiment in a distinct cohort of adult and adolescent, male and female, mice to examine marble-burying in the presence of a novel neutral versus novel aversive/anxiogenic odor (vanilla and tea tree, respectively). Based on the extant literature (Quadir et al., 2019), it was predicted that tea tree odor would increase marble-burying in all mice, which would suggest that the increased marble-burying observed in response to sex-related odors in Experiment 1 reflects an aversive response/anxiety-like behavior. However, if a presumably neutral odor such as vanilla (Yang & Crawley, 2009; Wersinger et al., 2007) also increased marble-burying, then the increased marble-burying in response to sex-related odors in Experiment 1 might also reflect a mere response to a novel smell. The experimental design is summarized in **Figure 3.1B**, with testing in the presence of the tea tree odor conducted on the third day out of concern that mice might develop a conditioned place-aversion that could negatively impact behavior.

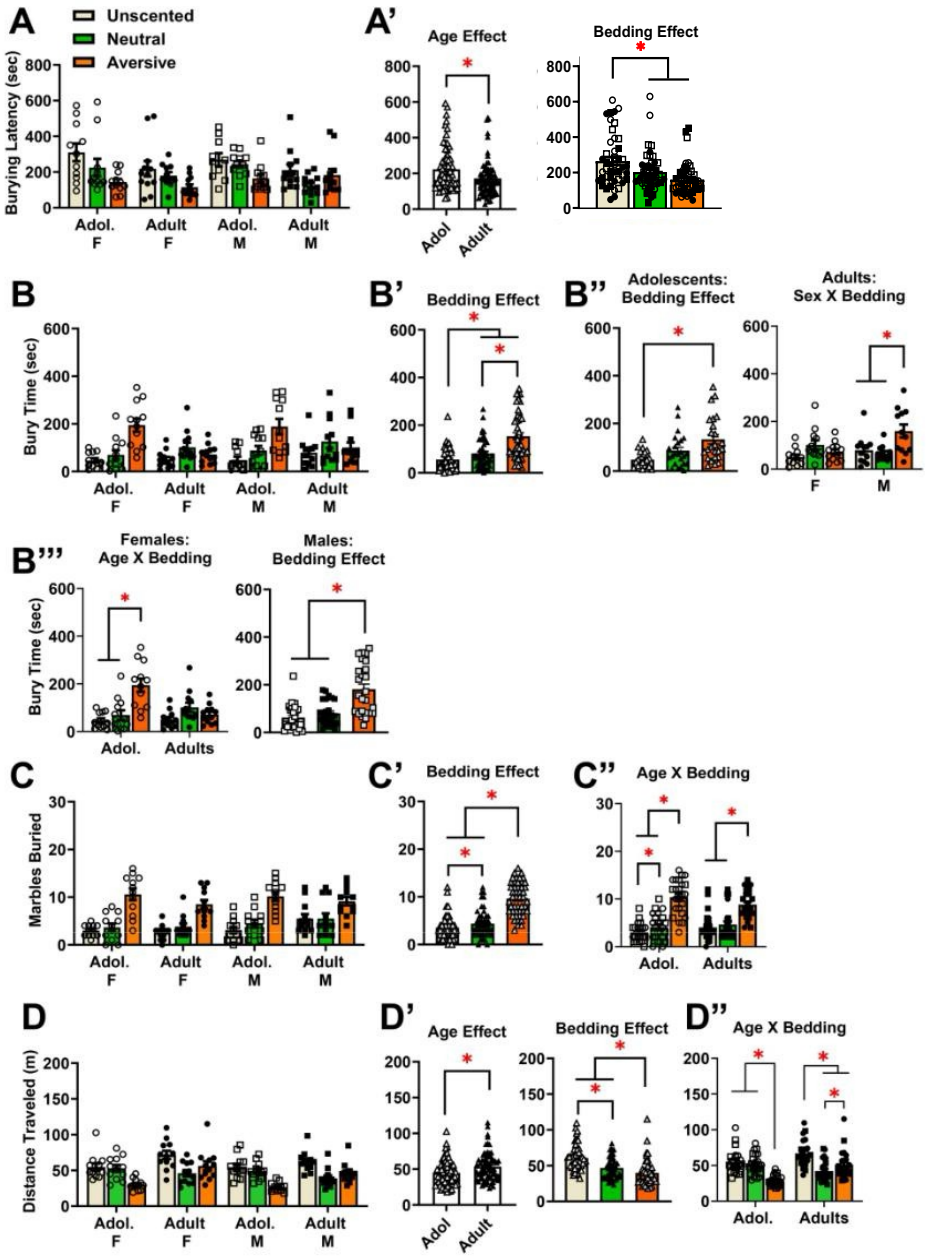


Figure 3.3. Summary of the effects of novel chemosensory stimuli on the marble-burying behavior of adolescent and adult mice (Experiment 2). (**A**) Summary of the latency to first begin marble-burying exhibited by the adolescent (Adol.) and adult male (M) and female (F) mice in the presence of unscented bedding or bedding scented with a neutral vanilla odor (Neutral) or a noxious tea tree odor (Aversive), for which a main Bedding effect was detected [females: Adol. unscented (n = 11), neutral-scented (n = 11), aversive-scented (n = 12); Adults unscented (n = 12), neutral-scented (n = 11), aversive-scented (n = 12); males: Adol. unscented (n = 10), neutral-scented (n = 11), aversive-scented (n = 12); Adults

unscented (n = 12), neutral-scented (n = 12), aversive-scented (n = 12)]. **(A')** Depiction of the significant Age and Bedding
Condition effects for the latency to bury. (**A"**) **(B)** Summary of the time spent burying by the mice in Experiment 2. **(B')** Depiction of the significant main Bedding Condition effect for the time spent burying. **(B")** Deconstruction of the significant 3-way interaction for the time spent burying along the Age factor revealed a significant Bedding Condition effect for adolescent mice and a significant Sex x Bedding interaction for adults. **(B"')** Deconstruction of the significant 3-way interaction along the Sex factor revealed a significant Age x Bedding interaction for female mice and a main Bedding Condition effect for males. **(C)** Summary of the number of marbles buried by the mice in Experiment. **(C')** Depiction of the main Bedding Condition effect for the number of marbles buried. **(C")** Depiction of the significant Age x Bedding interaction for this same variable. (**D**) Summary of the total distance traveled (in m) during Experiment 2. (**D'**) Depiction of the significant main effects of Age and Bedding condition for the distance traveled. (**D"**) Depiction of the significant Age x Bedding interaction for the distance traveled. The bar graphs represent the means ± SEMs of the number of individual mice indicated above. *$p < 0.05$ for indicated comparisons (LSD post-hoc tests).

3.3.2.1. Latency to Start Burying. A summary of the group differences in the latency to bury by the mice in Experiment 2 are presented in **Figure 3.3A**. In examining the factors influencing the latency to initiate marble-burying, we ran a mixed -model ANOVA with Age (adolescent vs. adult) and Sex (female vs. male) as between-subject factors, and Bedding Condition (unscented, neutral/vanilla, and aversive/tea tree) as a within-subjects factor. The results indicated no significant main effect of Sex [F(1,38) = 0.03, p = 0.869, eta = 0.001]. Furthermore, the Age x Sex x Bedding Condition interaction was also non-significant [F(2,76) = 0.65, p = 0.523, eta = 0.017]. No other significant interactions were observed [Sex x Bedding ANOVA: F(2,76) = 1.756, p = 0.180, eta = 0.044; Age x Bedding ANOVA: F(2,76) = 1.40, p = 0.254, eta = 0.035].

We did however detect a significant main effect of Age [F(1,38) = 5.48, p = 0.025, eta = 0.126], that reflected a shorter latency to start burying marbles in the adult versus adolescent mice (**Figure 3.3A', left**). The analysis also detected a significant main Bedding

Condition effect on the latency to start burying (**Figure 3.3A', right**) [F(2,76) = 8.55, p <.001, eta = 0.184]. As illustrated, LSD post-hoc tests indicated that this effect reflected a shorter latency to bury marbles in both the neutral vanilla (p = 0.030) and aversive tea tree scented bedding conditions (p <.001), relative to the unscented condition. Additionally, no significant overall difference was detected between the vanilla and tea tree-scented bedding (p = 0.066). These data for the latency to bury indicate that while adult mice are quicker to respond to the marbles than adolescent mice, irrespective of the odor present, novel odors of both neutral and aversive valence instigate marble-burying faster than unscented marbles, irrespective of the age or sex of the mouse.

3.3.2.2. Total time spent burying. A summary of the group differences in the total time spent burying by the mice in Experiment 2 are presented in **Figure 3.3B.** An Age x Sex x Bedding Condition (unscented, neutral/vanilla, and aversive/tea tree) mixed-model ANOVA was employed to examine for group differences in the total time spent marble burying. The results indicated no significant main effects of Sex [F(1,44) = 1.42, p = 0.239, eta = 0.031] or Age [F(1,44) = 1.76, p = 0.192, eta = 0.038] or interaction between these variables [F(1,44) = 0.68, p = 0.415, eta = 0.015]. A significant Bedding Condition effect [unscented < vanilla and tea tree (p's < 0.001); vanilla < tea tree (p = 0.035); F(2,88) = 34.45, p < 0.001, eta = 0.439] (**Figure 3.3B'**) as was the 3-way interaction [F(1,88) = 4.61, p = 0.013, eta = 0.095]. To investigate further, the ANOVA was first deconstructed along the Age factor to examine the Sex by Bedding Condition interaction separately for adolescents and adults. For the adolescent mice, the Sex x Bedding interaction was not significant [F(2,44) = 0.22, p = 0.807, eta = 0.010], however, a main effect of Bedding Condition was detected for this age

[F(2,44) = 31.17, p < 0.001, eta = 0.586], which is depicted in **Figure 3.3B" (left)**. LSD post-hoc tests indicated that adolescent mice buried more marbles in the tea tree-scented bedding, compared to the unscented bedding (p = 0.002), with no differences observed between vanilla-scented bedding and either the unscented (p = 0.235) or tea tree-scented conditions (p = 0.147). For the adults, a significant Sex x Bedding Condition interaction was observed [F(2,44) = 8.39, p < 0.001, eta = 0.276], which is depicted in **Figure 3.3B" (right)**. As illustrated, this interaction was driven by the male adult mice as tests for simple effects with LSD corrections did not detect a significant difference in the time spent burying by female mice in either the vanilla- (p = 0.052) or tea-tree-scented bedding (p = 0.213), compared to the unscented condition, while adult males spent more time burying in the tea tree-scented bedding versus both the unscented (p = 0.002) and vanilla-scented bedding (p = 0.009).

The significant 3-way ANOVA was then deconstructed along the Sex factor to explore age-related differences within each bedding environment within each sex. For females, the Age x Bedding Condition interaction was significant [F(2,44) = 14.14, p < 0.001, eta = 0.391]. As illustrated in **Figure 3.3B"' (left)**, subsequent analysis for simple main effects with LSD post-hoc corrections revealed that adolescent females spent more time burying in the tea tree-scented bedding, compared to both the unscented (p < 0.001) and vanilla-scented conditions (p < 0.001), with no difference in burying time between unscented and vanilla conditions (p = 0.339). In contrast, the time spent burying did not differ across bedding conditions in adult females (p's > 0.051). As illustrated in **Figure 3.3B"' (right)**, the Age x Bedding Condition analysis of the data for males indicated a main Bedding effect only [F(2,44) = 21.33, p < 0.001, eta = 0.492] that reflected a longer time burying by male

mice in the tea tree-scented bedding, compared to both the vanilla-scented ($p < 0.001$) and unscented condition ($p < 0.001$). These data for the time spent burying indicate that while both scented bedding conditions can augment burying behavior in adult and adolescent mice, tea tree odor produces a much more robust effect, consistent with its known aversive property.

3.3.2.3. Number of Marbles Buried. A summary of group differences in the number of marbles buried in Experiment 2 is provided in **Figure 3.3C**. An Age x Sex x Bedding Condition mixed-model ANOVA was conducted to examine for group differences in the number of marbles buried under the different scented conditions. The ANOVA results indicated no significant main effects of Age [$F(1,44) = 0.00$, $p = 0.982$, eta = 0.000] or Sex [$F(1,44) = 2.48$, $p = 0.12$, eta = 0.053], and no significant three-way interaction [$F(2,88) = 0.60$, $p = 0.554$, eta = 0.013]. A significant main effect was observed for Bedding Condition that is depicted in **Figure 3.3C'** [$F(2,88) = 86.60$, $p < .001$, eta = 0.663]. LSD tests for simple effects indicated that this Bedding Condition effect reflected more marbles buried under both the vanilla- ($p = 0.027$) and the tea tree-scented bedding ($p < 0.001$) versus the unscented condition, with mice also burying more marbles in the tea tree-than in the vanilla-scented bedding ($p < 0.001$). However, this effect varied as a function of age, as indicated by a significant Age x Bedding Condition interaction [$F(2,88) = 3.73$, $p = 0.028$, eta = 0.078]. Thus, this interaction was deconstructed along the Age factor to examine how the different bedding conditions influenced the number of marbles buried by adults and adolescent mice. As depicted in **Figure 3.3C''**, analysis of simple main effects with LSD corrections revealed that both the adolescent and adult mice buried more marbles in the aversive-scented tea tree

bedding when compared to both the neutral ($p < 0.001$ for both age groups) and unscented beddings ($p < 0.001$ for both age groups). Adolescent mice also exhibited increased burying behavior in the neutral vanilla bedding, compared to the unscented condition (**Figure 3.3C"**; $p = 0.049$). In contrast, adult mice showed no significant difference in marble burying between the neutral and unscented beddings (**Figure 3.3C"**; $p = 0.231$), indicative of an age-related difference in the response to the olfactory cues present in the bedding.

3.3.2.4. Distance Traveled. A summary of group differences in the distance traveled during the 20-min marble-burying sessions in Experiment 2 is provided in **Figure 3.3D**. We also employed an Age x Sex x Bedding Condition mixed-model ANOVA to investigate the locomotor activity of the mice, as measured by the distance traveled (in m) during the 20-minute marble-burying testing session. Analysis of the main effects failed to indicate a significant effect of Sex [$F(1,44) = 3.34$, $p = 0.074$, eta = 0.071]. However, significant effects of Age (Adult > Adolescent; **Figure 3.3D', left**) [$F(1,44) = 7.91$, $p = 0.007$, eta = 0.152] and Bedding Condition (**Figure 3.3D', right**) [$F(2,88) = 31.44$, $p < 0.001$, eta = 0.417] were detected. LSD post-hoc tests for simple effects indicated that the Bedding Condition effect reflected a larger distance traveled in the unscented bedding versus both the vanilla- ($p < 0.001$) and tea tree -scented bedding ($p < 0.001$), in addition to a larger distance traveled in the vanilla- versus tea tree-scented conditions ($p = 0.001$). Additionally, an interaction between these factors was also detected [$F(1.67, 73.25) = 17.62$, $p <.001$, eta = 0.286] that is depicted in **Figure 3.3D"**. Simple main effects analysis with LSD post-hoc corrections indicated that adolescent mice exhibited a marked reduction in locomotor activity when tested in the aversive tea-tree scented environment compared to the neutral vanilla and

unscented conditions (**Figure 3.3D"**: both p's < 0.001). In contrast, adult mice exhibited lower locomotion when tested in both scented conditions relative to the unscented bedding control (**Figure 3.3D"**; both p's < 0.001), although they did locomote more in the aversive tea-tree condition versus the neutral vanilla bedding condition (p = 0.004). No significant three - way interaction of Age x Sex x Bedding Condition was detected for the distance traveled in Experiment 2 [F(1.67, 73.25) = 17.62 , p <.001, eta = 0.286]. These data provide further evidence that locomotor activity is inversely related to marble-burying behavior, which likely reflects their behavioral incompatibility.

3.3.3. Experiment 3: Investigating aversive-conditioning following testing in the presence of an aversive odor

Considering the anxiogenic effect of exposure to tea tree oil observed in Experiment 2, we tested the hypothesis that initial exposure to tea tree odor might result in aversive-conditioning and increase subsequent marble-burying in the presence of clean, unscented, bedding. To this end, half of the mice underwent marble-burying procedures in the presence versus absence of the tea tree odor on day 1. The next day, all mice were tested under the standard, unscented condition to examine for carry-over effects as depicted in **Figure 3.1C**. Scoring of behavior was conducted manually for Experiment 3 due to a computer malfunction, precluding replay of behavior to determine the latency to bury, the time spent burying and the collection of locomotor activity data. Thus, only the data for the number of marbles buried is presented.

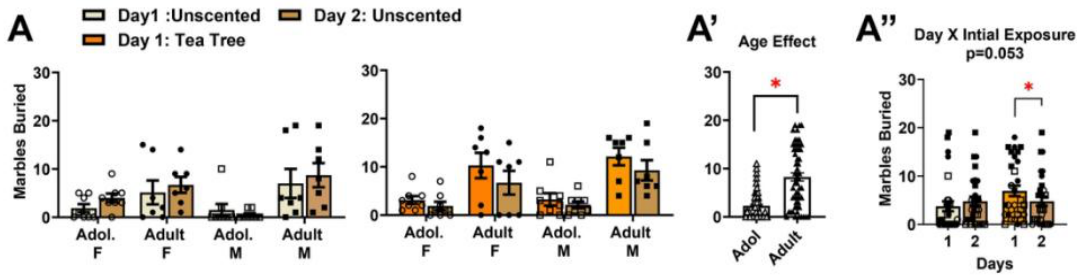


Figure 3.4. Examination of carry-over effects of a novel noxious chemosensory stimulus on the marble-burying behavior of adolescent and adult mice (Experiment 3). (A) Summary of the number of marbles buried by adolescent (Adol.) and adult male (M) and female (F) mice in the presence of unscented bedding **(left)** or tea tree-scented bedding **(right)** on day 1 of testing, and their response on day 2 of testing in the presence of unscented bedding only, the analyses of which yielded a strong trend towards a Day x Initial Exposure interaction [Adol. *n's* = 8/sex; Adults *n's*=7/sex]. **(A')** Depiction of the significant Age effect for the number of marbles buried. **(A")** Depiction of the strong statistical trend for a Day x Initial Exposure interaction. The bar graphs represent the means ± SEMs of the number of individual mice indicated above. *$p < 0.05$, Initial Exposure effect (t-test).

3.3.3.1. Number of Marbles Buried: A summary of the group differences in the number of marbles buried by the mice in Experiment 3 are presented in **Figure 3.4A.** The data were analyzed using a Day (day 1 vs. day 2) x Age (adolescent vs. adult) x Sex (female vs. male) x Initial Exposure (unscented bedding vs tea-tree scented bedding) ANOVA. This ANOVA indicated a significant main effect of Age (Adult > Adolescent; **Figure 3.4A'**) [F(1,42) = 25.06, p <.001, eta = 0.374], whereas the main effects of Day [F(1,52) = 0.98, p = 0.327, eta = 0.023, Sex [F(1,42) = 0.19, p = 0.665, eta = 0.005], and Initial Exposure [F(1,42) = 3.45, p = 0.070, p = 0.076] were not statistically significant.

Although the initial 4-way ANOVA failed to yield a significant interaction [F(1,42) = 0.06, p = 0.812, eta = 0.001], the data revealed a strong trend for a Day x Initial Exposure interaction [F(1,42) = 3.97, p = 0.053, eta = 0.086]. While we acknowledge that this

interaction was not statistically significant, the large effect size prompted us to explore the Day x Initial Exposure interaction. For this, the data was collapsed across both ages and sexes prior to its deconstruction along the Initial Exposure factor to examine how the initial exposure to tea tree oil impacted marble-burying the next day. As there were only 2 levels on the Day factor, t-tests were employed for this follow-up analysis. A comparison of the number of marbles buried by mice exposed twice to unscented bedding detected no change in marbles buried [**Figure 3.4A"**; $t(24) = 0.82$, $p = 0.421$]. In contrast, the number of marbles buried decreased when tea tree-exposed mice were allowed to bury marbles in unscented bedding (**Figure 3.4A"**) [$t(24) = 2.08$, $p = 0.048$]. This later result suggests that the anxiogenic effect of tea tree oil on the number of marbles buried does not persist, arguing against the formation of a conditioned place-aversion to the general cage testing environment (to include sawdust bedding).

3.4. Discussion

Our laboratory has spent nearly a decade examining age by alcohol interactions in the manifestation of negative affect during alcohol withdrawal, with most studies employing male mice exclusively (Szumlinski et al., 2023; Lee et al., 2015, 2016, 2017, 2018a-c). In these studies, we reliably report: (1) increased indices of anxiety-like behavior in adult mice during early (24 h) alcohol withdrawal; (2) an incubation of anxiety-like behavior in adolescent mice during protracted withdrawal; and (3) higher basal anxiety-like behavior in water-drinking adolescent versus adult controls (Lee et al., 2015, 2016, 2017, 2018a-c). However, the gender gap in binge-drinking is closing, particularly among adolescents (Squeglia et al., 2009, 2011) and the trajectory for developing an AUD occurs at a more rapid

rate in alcohol-drinking women than men (UNODC, 2023; Wilsnack et al., 2018; Agabio et al., 2017; Becker et al., 2017), with women (Grant et al., 2004; Hasin et al., 2007; Johnston et al., 2008; Kessler et al., 2005; Merikangas et al., 2007) and adolescent girls (Squeglia et al., 2009, 2011) exhibiting higher rates of comorbid psychiatric conditions, including affective disorders than age-matched males (Grant et al., 2004; Hasin et al., 2007; Johnston et al., 2008; Kessler et al., 2005; Merikangas et al., 2007). Thus, in recent years we have attempted to examine how biological sex moderates age-related differences in alcohol withdrawal-induced negative affect (Szumlinski et al., 2023). In contrast to our earlier studies, our more recent studies employing both sexes have failed largely at replicating our key observations (Szumlinski et al., 2023). Based on the results of a study by Kalaviers et al. (2001), indicating that female odors can have an anxiolytic effect on adult males exposed to predator threat, we questioned whether socially relevant, sex-related, olfactory cues might be masking or interfering with our ability to detect age-related differences in both basal and alcohol withdrawal-induced anxiety-like behavior. The present study was designed to begin to address this question by assaying for the influence of sex-related odors on marble-burying behavior in alcohol-naive male and female, adult and adolescent, mice. Below we discuss the present results in the context of the limited literature pertaining to the effect of innately motivating and novel benign olfactory cues on anxiety-like behavior in rodents.

Although female-soiled bedding did not influence the total number of marbles buried by the adult (PND 56-70) male mice in the present study, adult males exhibited a longer latency to begin, and a shorter time spent, burying marbles in the presence of female-related odors. These observations are interpreted as an anxiolytic effect of female pheromones in

adult male mice. Such findings are in line with the results of an earlier study of predator odor avoidance (Kavaliers et al., 2001), in which sexually inexperienced adult male mice showed fewer fear responses and less predator odor avoidance when pre-exposed (either 1 min or 30 min) to female odors. In the present study, all mice (including the mice from which the soiled bedding was obtained) were sexually naïve, and none of the mice had ever been directly exposed to a conspecific of the opposite sex. Thus, the anxiolytic effect of female odors on the behavior of sexually naïve males extends across at least two very distinct paradigms of affective motivation and it will be important to determine if female-related chemicals, whether volatile or non-volatile, exert similar anxiolytic effects in other popular assays of negative affect in male rodents (e.g., elevated plus maze, light-dark shuttle box, forced swim, and tail suspension tests). Gaining a clearer understanding of these effects would be of significant relevance to the experimental design and procedural timeline as well as the interpretation of findings from similar studies.

Admittedly, we did not monitor the estrous cycle of the females that generated the soiled bedding in this study. However, the female-soiled bedding was collected from multiple cages of group-housed females following a 6-day period and, thus, likely contained volatile and non-volatile chemicals emitted across all stages of the estrous cycle. Given our bedding collection procedure, it remains to be determined whether the apparent anxiolytic effect of the female-soiled bedding in adult males reflects a response to pheromones associated with female sexual receptivity that are innately motivating, e.g., (Been et al., 2019; Blanchard & Blanchard, 1977). However, it should be noted that in the prior predator odor avoidance study by Kalaviers et al. (2001), odors from sexually receptive and non-receptive females

evoked equivalent anxiolytic effects in sexually naïve males. Thus, the anxiolysis observed in adult sexually naïve males in the presence of female odors may be independent of the females' estrous phase, sexual receptivity, and/or sexual experience. Regardless of the specific female-related pheromones driving the anxiolytic effect of female-soiled bedding in adult males, the reduction in marble-burying does not likely reflect a novelty-induced suppression of burying behavior. This conclusion is based on the observation that adult males exhibited a shorter, not longer, latency to marble-bury in the presence of both the novel neutral vanilla and novel noxious tea tree odors in the follow-up studies.

In Experiment 1, adult males buried the most marbles when exposed to bedding soiled by unfamiliar males. As the male-soiled bedding was obtained from group-housed males, it is very likely that it contained a blend of pheromones from socially dominant, intermediate, and subordinate mice. However, male mice are highly territorial (Ralls, 1971), and indicate their territorial boundaries using urinary scent markings (Desjardins et al., 1973), with socially dominant males making urine marks in more locations and counter-marking more often than more subordinate males (Holekamp & Strauss, 2016; Jemiolo et al., 1985, 1991; Novotny et al., 1990). Thus, it can be presumed that of the four mice in each cage from which the male-soiled bedding was obtained, the majority of urine marks/pheromones present were those of the more dominant males. Based on the social dominance literature (Ralls, 1971; Desjardins et al., 1973; Holekamp & Strauss, 2016; Jemiolo et al., 1985, 1991; Novotny et al., 1990), such a scenario should elicit a negative affective state related to a potential threat of a resident attack, particularly given the inescapable nature of the marble-burying apparatus. As we did not establish social hierarchy

in our test subjects, it remains to be determined whether: (1) the greater propensity of males to bury marbles in male-soiled bedding reflects the relative amount of urine markings from dominant versus more subordinate males and (2) the social dominance of the male test subjects might dictate their response to the marbles in the presence of male- and/or female-soiled bedding, which could contribute to behavioral variability.

Although the anxiolysis exhibited by adult males in the presence of female-soiled bedding clearly does not require sexual experience to manifest, it does appear to depend upon their sexual maturity. This conclusion is based on the observation that young adolescent males (PND 27/28) exhibited higher, not lower, marble-burying in the presence of the female-soiled bedding. In fact, the magnitude of marble-burying by both adolescent males and females exposed to female- versus male-soiled bedding was equivalent. It was expected based on the social dominance literature, e.g., (Jemiolo et al., 1991), that adolescent mice of both sexes might exhibit more signs of anxiety-like behavior in response to an inescapable environment containing the odors/pheromones of more dominant, mature adult males. However, we did not necessarily anticipate higher signs of negative affect in adolescent males or females presented with female-soiled bedding. While the literature concerning female dominance and aggression is rather limited, c.f., (Ueno et al., 2020), female rats housed in same-sex colonies will determine dominant females that will attack any male intruder (Been et al., 2019), and social dominance among female Syrian hamsters is predicted by body weight in the context of both female-female and female-male interactions (Holekamp & Strauss, 2016). Furthermore, adult female mice will spend more time attacking younger versus older intruders (Arakawa et al., 2008). Thus, the increased marble-burying

exhibited by both adolescent males and females in the presence of female-soiled bedding could be driven by the potential threat of being attacked by an older resident female mouse.

Alternatively, the high level of marble-burying of the adolescent males and females in response to both male- and female-soiled bedding may reflect their general responsiveness to novel odors. Although all mice exhibited a shorter latency to begin marble-burying in the presence of the neutral vanilla odor, only the adolescent mice buried more marbles in this context relative to the unscented, standard condition. Based on prior studies of the innate motivating properties of the two novel scents employed in this study (Yang & Crawley, 2009; Wersinger et al., 2007; Quadir et al., 2019), the number of marbles buried by adolescent mice in the presence of the vanilla odor was significantly less than that in the presence of the noxious tea tree odor, arguing that the tea tree odor is more anxiogenic than the vanilla odor in adolescent mice. Nevertheless, while age-related differences in marble-burying under the standard, unscented condition were not consistently detected across our three experiments, they did manifest in the presence of a male- and female-related odors, as well as a novel neutral odor. This suggests that the olfactory context is an important determinant of the ability to detect subject factor effects and interactions in this assay. Conversely, the fact that all mice exhibited similarly robust increases in marble-burying behavior in the presence of tea tree oil raises the possibility that the presence of novel noxious odorants (plant-based or otherwise) might mask the ability to detect subject factor differences and interactions in behavior. An important consideration for future research is whether our findings with the novel odorant extend to chemicals employed to sanitize the test

cages and/or mask the scent of other test subjects (e.g., 70% (v/v) ethanol solutions or virucidal agents).

As observed in adult males, the presence of bedding soiled by same-sex conspecifics increased marble-burying in adult female mice as indicated by more time spent burying and more marbles buried, relative not only to the standard, unscented condition, but also to the male-soiled condition. Given the evidence for a social dominance hierarchy in group-housed female rats (Been et al., 2019), it is possible that the high marble-burying exhibited by adult females in the presence of female-soiled bedding might be driven by the potential threat of being attacked by a more dominant conspecific.

Alternatively, the adult female response might reflect the threat of resource competition emanating from the collection of pheromones from multiple unfamiliar female mice. Competition under conditions of limited resources (including food, water, nesting materials, sexual partners) is known to increase aggressive tendencies toward other females in rodents (Been et al., 2019; Holekamp & Strauss, 2016; Arakawa et al., 2008), but it is not known whether the threat of resource competition might induce a negative affective state in either female or male mice. As females are attracted to testosterone-dependent volatiles in male urine that indicate social status (Jemiolo et al., 1985, 1991; Novotny et al., 1990), it is perhaps not surprising that male-soiled bedding elicited a less robust effect on marble-burying by adult female mice than female-soiled bedding.

The facts that odors from same-sex adult conspecifics and odors from older animals increase marble-burying in adult and adolescent mice, respectively, in a manner akin to a noxious odorant speaks to the powerful influence exerted by chemosensory cues on anxiety-related behavior, particularly when studying same-sex cohorts of animals. That odors related to the opposite sex exert effects on the baseline behavior of adult mice that either are weaker (for females) or opposite (for males) to those effects produced by same-sex conspecifics also demonstrates that chemosensory stimuli shift baseline behavior in a sex- and age-dependent manner. Although the present study is not explicitly designed to delineate the nuanced impacts of social chemosensory stimuli on alcohol-induced changes in marble-burying behavior, it is relevant for interpreting the negative results from our prior studies of binge-drinking and negative affect (Szumlinski et al., 2023). Specifically, our current results highlight the potential for social chemosensory cues, and possibly novel noxious odors from cleaning products, to minimize, completely obscure, or even facilitate the detection of subject factor differences, at least in the marble-burying assay.

3.5. Conclusions

Behavior expressed in the marble-burying test is influenced by the presence of odors in the test environment. The direction and magnitude of the influence of sex-related odors from both males and females, novel neutral odors (e.g., vanilla scent) and novel noxious odors (e.g., tea tree oil) varies as a function of the biological sex and/or the developmental age of the mouse.

Chapter 4:

A subchronic history of binge-drinking elicits mild, age- and sex-selective, affective, and cognitive anomalies in C57BL/6J mice

4.1. Introduction

One of the most common risk factors for the development of dementia and cognitive decline is a history of alcohol abuse (Schwarzinger et al., 2018; Nunes et al., 2019; Wiegmann et al., 2020). Numerous studies have identified that both alcohol use disorder (AUD) and dementia, particularly Alzheimer's Disease (AD), have a high incidence of cooccurrence (Hersi et al., 2017; Hoffman et al., 2019). Recent evidence suggests that excessive drinking may play a significant role in the development of early-onset dementia and related disorders (Piazza-Gardner et al., 2013; Heymann et al., 2016; Huang et al., 2018; Ledesma et al., 2021). According to evidence from both rodent and human studies, repetitive binge-drinking episodes throughout adolescence are sufficient to generate disruptions within the mesocorticolimbic system that may cause long-term deficiencies in emotional regulation and poor cognitive abilities that become apparent later in adulthood (Novier et al., 2015; Cservenka and Brumback, 2017).

Characterized as stage of rapid neurodevelopment, adolescence normally takes place between 12 and 17 years of age in humans and 28–50 postnatal days (PND) in laboratory mice. Adolescence is commonly recognized as a transitional period marked by the onset of puberty and accompanied by rapid neurobiological, social, and cognitive development (Spear, 2000a,b). As a result of these changes, heightened risk-taking is a hallmark characteristic of adolescence that contributes to the incidence and prevalence of substance use disorders, including AUD (Steinberg, 2007; MacPherson et al., 2010). In contrast to adults, adolescents also exhibit milder affective disturbances and are less vulnerable to the sedative and cognitive deficits that often occur during alcohol withdrawal (Varlinskaya and

Spear, 2004; Lee et al., 2016). Thus, research suggests that the perceived advantages of binge-drinking are often more pronounced during this age and such an age-specific attenuation in sensitivity to alcohol's aversive properties may serve as a permissive factor that contributes to the maintenance of binge drinking patterns among adolescents (Varlinskaya and Spear, 2004; Spear and Varlinskaya, 2005).

The motivational factors that drive drinking to intoxication differ between biological sexes in both humans and laboratory rodents. Evidence suggests that human females are more likely to engage in alcohol binge-drinking to alleviate physical and psychological distress, compared to males (Rodriguez et al., 2020). Although both sexes report a high rate of comorbid mood disorders with AUD, females demonstrate a heighted susceptibility to both the psychological and physiological consequences of excessive drinking (Pollard et al., 2020; Rodriguez et al., 2020). Further, a few findings allude to the notion that females with a history of alcohol abuse experience earlier and greater cognitive-behavioral impairments than their male counterparts (Hebert et al., 2013; Agabio et al., 2017; Ferretti et al., 2018). While several hypotheses attempt to explain why females experience more severe biopsychological effects than males because of alcohol, there is relatively little research that directly examines for sex differences in the effects of excessive drinking on affect or cognitive function, let alone how the age of drinking-onset might interact with biological sex to impact the severity of affective and/or cognitive disturbances during alcohol withdrawal.

Toward this end, we published a study in 2020 designed to examine for sex by age interactions in the expression of negative affect during early (1 day) versus protracted (70

days) alcohol withdrawal in C57BL/6J (B6) mice (Jimenez Chavez et al., 2020). In contrast to other published findings from our laboratory that studied a single sex (e.g., males: Lee et al., 2015, 2016, 2017b, 2018a,b; females: Szumlinski et al., 2019), we detected relatively few behavioral signs of alcohol withdrawal-induced anxiety-like behavior, irrespective of the age of binge-drinking onset. However, when effects of alcohol withdrawal were detected, the magnitude of the effect was comparable between male and female subjects. Two procedural differences might account for the discrepancies in findings between our study of sex differences (Jimenez Chavez et al., 2020) and those employing a single sex (Lee et al., 2016, 2017a,b, 2018a,b; Szumlinski et al., 2019). The first relates to the duration of the alcohol withdrawal period as earlier work compared anxiety-like behavior between 1- and 30-days withdrawal and showed that (at least in adult male B6 mice with a 2-week history of binge-drinking) signs of negative affect dissipate by the 30-day withdrawal time-point (Lee et al., 2017b, 2018a). In contrast, some signs of alcohol-induced negative affect persist for at least 30 days in adult female B6 mice (Szumlinski et al., 2019), but may dissipate at some time between 30 and 70 days withdrawal (Jimenez Chavez et al., 2020). The second procedural difference relates to the concurrent testing of males and females and the potential for sex-related pheromones to influence the affective responses of mice of the opposite sex. Indeed, chemosensory social stimuli, such as those in vaginal secretions, are reported to alter neuronal activity within the mesocorticolimbic system differentially in adolescent versus adult males to affect motivated behavior (Romeo et al., 1998; Bell et al., 2013a,b). Further, exposure to adult female urinary pheromones during testing for anxiety-like behavior produces a testosterone-driven anxiolytic effect in male rats and mice (Aikey et al., 2002; Fernández-Guasti and Martínez-Mota, 2005; Frye et al., 2008). While it is known that

affective behavior varies with the estrous cycle in adult female rodents (Fernandez-Guasti and Picazo, 1992), to the best of our knowledge, there is no published report examining how exposure to adult male pheromones might alter anxiety-like behavior in female rodents.

The present study attempted to address both procedural issues by staggering binge-drinking procedures so that anxiety-like behavior was assayed separately in male and female mice on withdrawal days 1 and 30 (respectively, WD1 and WD30). As recent work indicated that mature adult females are more sensitive than their male counterparts to alcohol-induced cognitive impairment (Jimenez Chavez et al., 2022), mice in this study then underwent training under Morris water maze and radial arm water maze procedures to examine for sex by age interactions in alcohol-induced deficits in spatial and working memory in younger adult mice (see **Figure 4.1**). Based on the current literature (Szumlinski et al., 2019; Ledesma et al., 2021; Jimenez Chavez et al., 2022), it was hypothesized that alcohol-induced changes in affective and cognitive behavior would be more pronounced in females than males and that a history of binge-drinking during adolescence would induce more robust and/or enduring changes in behavior than that produced by a history of binge-drinking during adulthood.

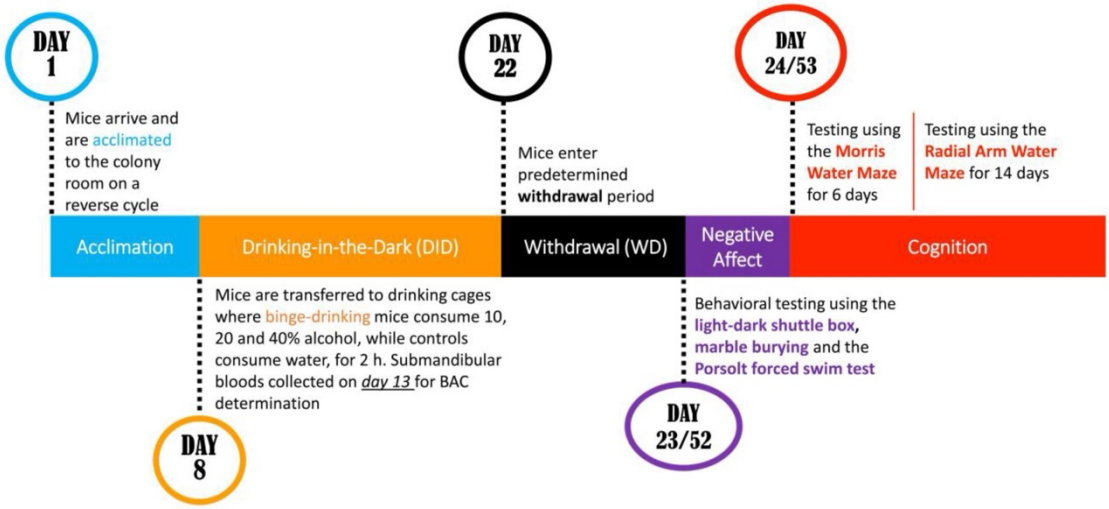


Figure 4.1. Cartoon of the procedural timeline of the experiments conducted in the present study.

4.2. Materials and Methods

4.2.1. Subjects

This experiment employed adolescent (postnatal day; PND 21) and adult (PND 49), male and female B6 mice sourced from The Jackson Laboratory (Sacramento, CA, United States). Upon arrival to the vivarium, the mice were immediately housed in groups of four with others of the same age and sex. Mice were allowed 7 days to acclimate to a colony room in a temperature-controlled vivarium under a 12-h reverse light/dark cycle (lights off at 10:00 h). To accommodate space constraints in our vivarium and testing facility, the mice in both withdrawal groups were subdivided into two cohorts, each cohort with a relatively equal number of animals in each group, matched for age, sex and drinking history. In the first cohorts, male mice began drinking a day before the female mice, to ensure that males and females were tested for anxiety-like behavior on different days, thereby minimizing the influence of chemosensory stimuli from the opposite sex; the inverse was done on the

subsequent cohorts (Jimenez Chavez et al., 2020, 2022). All animals were identified via tail markings, with access to food and water ad libitum, except during the 2-h alcohol-drinking session. In accordance with standard vivarium protocols, drinking cages were lined with sawdust bedding. To minimize any external stressors from unfamiliar handling and changes in the environment, routine cage cleaning activities were halted 5 days before behavioral testing. All experimental methods remained complaint with The Guide for the Care and Use of Laboratory Animals (2014) and all protocols were approved by the Institutional Animal Care and Use Committee of the University of California, Santa Barbara.

4.2.2. Drinking-in-the-Dark (DID)

A total of 92 mice were subjected to 14-days of binge drinking using a multi-bottle DID procedure that involved concurrent access to unsweetened 10, 20, and 40% (v/v) ethanol. At 13:00 h, all alcohol-drinking mice were relocated from their home cages to individual drinking cages, fitted with a wire lid, located on a free-standing rack in the same colony room within the vivarium. All animals were given 1 h to habituate to their drinking cages prior to alcohol presentation. At 14:00 h, the binge-drinking mice were provided with three alcohol-containing sipper tubes atop the wire cage lid for 2 h (14:00 h–16:00 h), with the position of the sipper tubes randomized each day. As a result of limited space on the freestanding rack, the water-drinking control mice were group housed in drinking cages with their cage mates and received one sipper tube containing water as conducted in comparable studies (e.g., Lee et al., 2018b; Szumlinski et al., 2019; Jimenez Chavez et al., 2020, 2022). Following the 2-h drinking session, all sipper tubes were removed, and the mice were transferred back into their respective home cages. The alcohol-containing sipper tubes were

weighed to determine individual consumption. Throughout the drinking period, mice were weighed every 4 days and their weights were utilized to calculate their overall alcohol intake.

4.2.3. Blood Alcohol Concentrations

On the 13th day of drinking, submandibular blood samples were collected from the alcohol-drinking mice immediately following their 2-h drinking session. Analytical methods for determining blood alcohol concentrations (BAC) are similar to those employed in our previous studies (Fultz and Szumlinski, 2018; Jimenez Chavez et al., 2020, 2022). Blood samples were stored at −20°C until processing and BACs were determined using headspace gas chromatography. The analysis was performed using a Shimadzu GC-2014 gas chromatography system (Shimadzu, Columbia, MD, USA), and the data was obtained using the GC Solutions 2.10.00 software. To determine the alcohol concentration in each sample, the samples were diluted with non-bacteriostatic saline at a ratio of 1:9, with 50 µl of the sample, and toluene was used as the pre-solvent. The analysis was conducted within 7–10 days of sample collection.

4.2.4. Behavioral Test Battery for Negative Affect

Evidence from our prior work indicated that adolescent male B6 mice with a 2-week history of binge-drinking do not display any noticeable signs of negative affect when tested at 1 day withdrawal (e.g., Lee et al., 2016, 2017b, 2018a,b; Szumlinski et al., 2019; Jimenez Chavez et al., 2020, 2022), we conducted a comprehensive 1-day behavioral test battery including the light-dark shuttle box test, the marble burying test and the Porsolt forced swim test to measure withdrawal-induced changes in negative affect, as detailed below. The order

of testing in the various procedures was pseudo-randomized, except for the forced swim test, which occurred last in the test battery in accordance with our animal use protocol. To mitigate any potential impact of chemosensory stimuli from the opposite sex on behavior, we tested males and females on separate days.

4.2.4.1. Light–Dark Shuttle Box Test

The light-dark shuttle box test is a behavioral paradigm employed in preclinical research to evaluate anxiety-like behaviors in rodents (Crawley, 1985; Bourin and Hascoët, 2003). In this test, mice are placed in the dark side of a polycarbonate box (46 cm × 22 cm × 24 cm) comprised of two distinct (light vs. dark) environments of equal areas. The light side of the box was white with no lid, while the dark side was black with a black lid. A central divider with an opening allowed the mice to access both sides throughout the 5-min test. The behavioral indices of anxiety-like behaviors, including latency to enter the light side, total time spent in the light side, and the total number entries to the light side, were measured using AnyMaze tracking software (Stoelting Co., Wood Dale, IL, USA). After each testing session, the apparatus was disinfected with Rescue Disinfectant Veterinary Wipes (Virox Animal Health, Oakville, ON, Canada) and the mice were returned to their home cages.

4.2.4.2. Marble Burying Test

The marble burying test is an established rodent behavioral paradigm that is sensitive to alcohol withdrawal-induced changes in negative affect (Lee et al., 2016, 2017a,b; Szumlinski et al., 2019; Jimenez Chavez et al., 2020, 2022). Mice were placed in a polycarbonate box (12 cm × 8 cm × 6 cm) filled with sterilized sawdust bedding 5 cm deep

and 20 round glass black marbles arranged equidistantly in a 4 × 5 square pattern. Animals were allowed to explore the environment and bury marbles for 20 min, where more burying behaviors indicated increased negative affect. After each session, the total number of marbles buried was tallied by the experimenter, the sawdust bedding was replaced with clean bedding, and the mice were returned to their home cages.

4.2.4.3. Porsolt Forced Swim Test

The Porsolt forced swim test is a behavioral paradigm often used to evaluate the reversal of passive coping behavior by antidepressant therapies (Porsolt et al., 2001). The increased swimming behavior observed in this assay can be reversed by pretreatment with anxiolytic agents (Lee et al., 2017a) and therefore, we incorporated it as an additional measure of negative affect. In this assay, mice were placed into a cylindrical glass container (11 cm in diameter) filled with room temperature water for 6 min. Using the AnyMaze tracking software, we measured the latency to the first immobile episode, the total time the animal was immobile, and the number of immobile episodes. Following completion of the test, the mice were returned to their home cages and were monitored until they were dry before returning to the colony room.

4.2.4.4. Morris Water Maze

Following the 1-day test battery for negative affect, conducted on either withdrawal day 1 (WD1) or 30 (WD30), all mice underwent a Morris water maze procedure to assay spatial learning and memory (see **Figure 4.1**). The Morris water maze procedures were like those employed previously by our group, using digital video-tracking and AnyMaze software

(Lominac et al., 2005; Datko et al., 2017; Jimenez Chavez et al., 2022). The maze is a stainless-steel circular tank (200 cm x 60 cm) containing black intra-maze cues (sun, checkerboard, stripes, moon) one at each four compass coordinate points (N,S,W,E). The tank was filled with room temperature water such that the water level was just above the top of the clear, glass, escape platform. On the first day, a "flag test" was conducted that assayed for visually cued spatial navigation and examined for group differences in swimming speed. For this, a red flag, extending 6 inches above the water, was attached to the escape platform so that the platform location was visible to the mice and the platform was positioned in the NW quadrant. The mice were allowed 2 min to locate the platform and were returned to their home cage upon platform location. If a mouse failed to locate the platform, additional 2-min sessions were conducted until the mouse located the flagged platform. The subsequent 4 days consisted of maze acquisition training, during which the flag was removed and the hidden platform remained situated in the NE quadrant. During acquisition, mice were released from one of the four compass points and allowed 2 min to locate the hidden platform. Once found, mice remained on the platform for 15 s, prior to being returned to the home cage. Once all of the mice completed the first compass point, they were released from the other three compass points so that each mouse underwent four 2-min trials per day. If a mouse failed to locate the hidden platform at any point during maze acquisition, it was guided gently to the platform using long forceps and remained on the platform for 15 sec prior to being returned to the home cage. Twenty-four hours following the fourth acquisition training day, a "probe test" was conducted in which the hidden platform was removed from the tank, and mice swam freely for 2 min and the time spent swimming in the NE quadrant that formerly contained the platform was recorded to index spatial recall. The day following the probe test, a reversal test

was conducted in which the hidden platform was positioned in the SW quadrant (i.e., the quadrant opposite to that employed during maze acquisition), and mice underwent four 2-min trials (one for each compass point) in which they were to find the new platform location.

4.2.4.4. Water Version of the Radial Arm Maze

Following a 1–2 day break, mice were then trained to locate 4 hidden platforms in a water version of the radial arm maze to evaluate working and reference memory. Akin to prior studies (Lominac et al., 2005; Szumlinski et al., 2005; Jimenez Chavez et al., 2022), the maze featured eight arms, four of which had underwater platforms, with the platform locations remaining constant throughout the 14-day training period, but varied for each mouse. Each mouse underwent four, 3-min, trials per day and the trials were conducted in series until the mouse located all four hidden platforms. Upon location of a hidden platform, the mouse remained on the platform for 15 s, at which time it was transferred to a heated holding cage for a 30-s period and the platform was removed from the maze. This was repeated until all four platforms were located. Trained researchers observed the mice throughout each 3-min trial and documented their arm entries in order to calculate the number of reference errors (first entry into an arm that never contained a platform; total of 4 possible), the number of working memory correct errors (entries into an arm that previously contained a platform), the number of working memory incorrect errors (repeated entries into an arm that never contained a platform), chaining behavior (consecutive entries into adjacent arms, irrespective of platform location; a non-spatial navigation strategy) and the time required to locate the platform. The first day of testing was considered a training day and thus was excluded from statistical analysis. The number of each type of error, the number of

chains and the time taken during each trial were each summed across the four trials to provide a total for each variable for each training day.

4.2.5. Replicate Study of Withdrawal-Induced Negative Affect

The results of the large-scale study described above yielded relatively few signs of alcohol withdrawal-induced negative affect. As assays were conducted concurrently with other testing, we attempted to reduce the influence of any concurrent testing and related personnel traffic in a replicate study more in line with prior studies by our group (e.g., Lee et al., 2016, 2018a,b; Szumlinski et al., 2019). We also single-housed the water-drinking controls during drinking procedures to equate the daily 3-h periods of social isolation across the drinking groups. Otherwise, the drinking and behavioral testing procedures for this replicate study were identical to those employed in the larger scale study described above. Again, males and females were tested for anxiety-like behavior on different days to avoid chemosensory cues from the opposite sex. As the withdrawal-induced negative affect exhibited by adult mice in early withdrawal is robust according to our earlier studies (Lee et al., 2016, 2017a,b; 2018a,b; Szumlinski et al., 2019), we opted to examine behavior at this time-point only in this replicate study with two expectations: (1) adolescent water controls would exhibit more anxiety-like behavior than adults and (2) adult, but not adolescent, alcohol-drinking mice would exhibit signs of anxiety-like behavior. Based on recent work (Jimenez Chavez et al., 2020, 2022), coupled with the majority of results from the present large-scale study (see "Results"), we did not predict any sex difference in the manifestation of withdrawal-induced negative affect. Thus, we employed a sample size of n = 6/sex/age/drinking history.

4.2.6. Statistical Analysis

To ensure comparable alcohol intake and BECs between the groups of mice slated to be tested on withdrawal day 1 versus withdrawal day 30 (respectively, WD1 versus WD3), these variables were analyzed using a Sex × Age × Withdrawal ANOVA. The data for alcohol intake in the replicate study was analyzed using a Sex × Age ANOVA. Previous findings from our laboratory suggest that the magnitude of alcohol withdrawal-induced negative affect is influenced by the length of withdrawal (Lee et al., 2016, 2017b, 2018; Szumlinski et al., 2019; Jimenez Chavez et al., 2020). Therefore, to reduce the complexity of the statistical analyses and increase interpretability of the results from the large-scale study, the data for our measures of negative affect and cognitive function were analyzed separately for early (starting on WD1) and late (starting on WD30) withdrawal using a Sex × Age × Drinking History ANOVA. Alpha was set at 0.1 for all analyses as we had a priori predictions that: (1) adolescent water-drinking mice would exhibit higher baseline emotionality than their adult counterparts (Lee et al., 2016, 2017a,b); (2) adult binge-drinking mice would exhibit robust signs of negative affect, particularly on WD1 (Lee et al., 2015, 2016, 2017b, 2018a,b; Szumlinski et al., 2019; Jimenez Chavez et al., 2022); and (3) signs of alcohol withdrawal-induced negative affect expressed by adolescent-onset binge-drinkers would be more robust on WD30 compare to WD1 (Lee et al., 2016, 2017b, 2018a,b). For the cognitive data, we conducted Sex x Age x Drinking ANOVAs, with the repeated measures variables of Day/Trial, when appropriate. To increase the statistical power to identify lower-level age and sex differences in our cognitive measures, alpha was set at 0.05 for all analyses and post hoc LSD comparisons were performed. For all analyses where sphericity was

violated, a Greenhouse–Geisser correction was used. Outliers were identified and excluded from the analyses using the ± 1.5 × IQR rule, however, in instances were too many outliers were identified, we adopted the ± 3 × IQR rule to ensure that only the most extreme outliers were removed. IBM SPSS Statistics software (version 27.0 for Macintosh) was used for all statistical tests, and GraphPad Prism software (version 9.3.1 for Macintosh) was used to create all graphs.

In addition to our primary analyses employing a general linear model, we sought to enhance the comprehensiveness of the data analysis for the large-scale study by employing generalized linear models (GLMs) for our between-subjects analyses. Within this framework, we selected specific GLM types provided by SPSS that were suitable for the nature of our response variables. GLMs are particularly used when assumptions underlying traditional general linear models are violated, allowing for a more flexible modeling approach that adapts to various data distributions and response types (Neal and Simons, 2007; Ng and Cribbie, 2017). For continuous (scale) responses, we implemented two GLM variations: (1) a linear GLM with a normal distribution assumption and the identity link function, and (2) a gamma GLM with a gamma distribution assumption and the logarithmic link function. For count-based response variables, we employed (1) a Poisson loglinear GLM assuming a Poisson distribution and the logarithmic link function, and (2) a negative binomial GLM assuming a negative binomial distribution and the logarithmic link function. Finally, for the dependent variable measuring the number of marbles buried, we utilized a binary logistic GLM with a binomial distribution assumption and the logit link function, as well as a Poisson loglinear GLM. The binary logistic GLM was chosen due to the variable's bounded

maximum value of 20 marbles. Overall, these additional analyses remained consistent with the results from the general linear model (3-way ANOVA; see **Tables 4.1–4.3**)

Withdrawal day 1						
Dependent variable	General linear model		Generalized linear model (Gamma)		Generalized linear model (Linear)	
	Interaction	*P*-value	Interaction	*P*-value	Interaction	*P*-value
Latency to enter the light side	None	all *p*'s > 0.160	None	all *p*'s > 0.169	None	all *p*'s > 0.139
Time spent in the light side	Age × DID Sex × Age Age Effect Sex Effect	0.012 0.040 0.066 0.009	Age × DID Sex × Age Age Effect Sex Effect	0.011 0.022 0.049 0.008	Age × DID Sex × Age Age Effect Sex Effect	0.007 0.029 0.052 0.005
Latency to immobility	DID effect	0.040	DID effect	0.025	DID effect	0.029
Time spent immobile	3-way Inx. Age Effect	0.047 0.019	3-way Inx. Age Effect	0.063 0.027	3-way Inx. Age Effect Sex Effect	0.034 0.012 0.095
Flag test time	None	all *p*'s > 0.500	None	all *p*'s > 0.553	None	all *p*'s > 0.479
Latency to enter platform area	Age × DID	0.021	Age × DID Sex × Age	0.007 0.056	Age × DID	0.014
Time in the probe test	None	all *p*'s > 0.221	None	all *p*'s > 0.215	None	all *p*'s > 0.198
Withdrawal day 30						
Latency to enter the light side	None	all *p*'s > 0.228	None	all *p*'s > 0.247	None	all *p*'s > 0.204
Time spent in the light side	None	all *p*'s > 0.140	None	all *p*'s > 0.162	None	all *p*'s > 0.119
Latency to immobility	Sex Effect	0.006	Sex Effect	0.004	Sex × DID Sex Effect	0.084 0.003
Time spent immobile	Sex × DID Age Effect Sex Effect	0.072 0.032 0.003	Sex × DID Age Effect Sex Effect	0.059 0.033 0.003	Sex × DID Age Effect Sex Effect	0.057 0.022 0.002
Flag test time	None	all *p*'s > 0.222	None	all *p*'s > 0.222	None	all *p*'s > 0.199
Latency to enter platform area	None	all *p*'s > 0.461	None	all *p*'s > 0.441	None	all *p*'s > 0.281

Table 4.1 Comparative analysis of significant statistical results on continuous data for the measures of negative affect and cognition using a general linear model, gamma generalized linear model with log link function (Gamma), and linear generalized linear model (Linear).

Withdrawal day 1						
Dependent variable	General linear model		Generalized linear model (Poisson loglinear)		Generalized Linear model (Negative binomial)	
	Interaction	*P*-value	Interaction	*P*-value	Interaction	*P*-value
Entries to the light side	Age × DID Sex × DID	0.032 0.062	Age × DID Sex × DID	0.033 0.065	None	all *p*'s > 0.621
Immobile episodes	3-way Inx. Age Effect	0.005 0.034	3-way Inx. Age Effect Sex Effect	<0.001 <0.001 0.036	None	all *p*'s > 0.256
Entries to platform area	None	all *p*'s > 0.386	None	all *p*'s > 0.286	None	all *p*'s > 0.710
Withdrawal day 30						
Entries to the light side	Sex Effect	0.003	3-way Inx. Age x DID Sex Effect	0.086 0.098 <0.001	None	all *p*'s > 0.352
Immobile episodes	Age × DID Sex × DID Sex Effect	0.094 0.052 0.007	Age × DID Sex × DID DID Effect Sex Effect	0.044 0.011 0.076 <0.001	None	all *p*'s > 0.402
Entries to platform area	Age × DID	0.012	Age × DID	0.001	None	all *p*'s > 0.280

Table 4.2. Comparative analysis of significant statistical results on count data for the measures of negative affect and cognition using a general linear model, poisson generalized linear model with log as the link function (Poission loglinear), and negative binomial generalized linear model with log as the link function (Negative binomial).

Withdrawal day 1						
Dependent variable	General Linear model		Generalized Linear model (Binary logistic)		Generalized Linear model (Poisson loglinear)	
	Interaction	*P*-value	Interaction	*P*-value	Interaction	*P*-value
Number of marbles buried	Age Effect	0.048	Age Effect Sex Effect	<0.001 0.045	Age Effect	0.003
Withdrawal day 30						
Number of marbles buried	Age Effect Sex Effect	0.055 0.002	3-way Inx. Sex × DID DID Effect Age Effect Sex Effect	0.002 0.025 0.041 <0.001 <0.001	3-way Inx. Sex × DID DID Effect Age Effect Sex Effect	0.013 0.068 0.072 0.006 < 0.001

Table 4.3. Comparative analysis of significant statistical results on count data for the number of marbles buried in the marble burying test using a general linear model, binary logistic generalized linear model with logit as the link function (Binary logistic), and poisson generalized linear model with log as the link function (Poisson loglinear).

4.3. Results

4.3.1. Alcohol Intake and BECs

A univariate Sex × Age × Withdrawal ANOVA was conducted to determine group differences in the amount of alcohol consumed during the 14 days of drinking and to confirm equivalent intakes between mice slated to be tested for behavior on WD1 and WD30. While a statistically significant main effect of Withdrawal was observed [$F(1,84) = 3.99$, $p = 0.049$, $\eta 2p = 0.045$], its practical significance may be limited due to the relatively weak effect size and the unequal sample sizes in our study. As such, the data are presented as collapsed across the two withdrawal time-points in **Figure 4.2**. Adolescent mice exhibited higher alcohol intake than adult mice [**Figure 4.2A**; Age effect $F(1,84) = 45.491$, $p < 0.001$, $\eta 2p = 0.351$], as well as higher alcohol intake by female mice than males [**Figure 4.2A**; Sex effect $F(1,84) = 40.326$, $p < 0.001$, $\eta 2p = 0.324$]. No significant 3-way interaction was observed for the average alcohol intake ($p = 0.754$, $\eta 2p = 0.001$) and no other significant interactions were observed (all *p's* > 0.066). The average BEC attained on Day 13 of drinking (**Figure 4.2B**) exhibited a pattern of group differences that was comparable to that of the average alcohol intake of the mice [Age effect: $F(1,62) = 15.05$, $p < 0.001$, $\eta 2p = 0.195$; Sex effect: $F(1,62) = 10.06$, $p = 0.002$, $\eta 2p = 0.140$] and consistent with this, a Pearson's correlation showed a positive relationship between BEC levels and alcohol intake ($r = 0.59$, $p < 0.001$, **Figure 4.2C**).

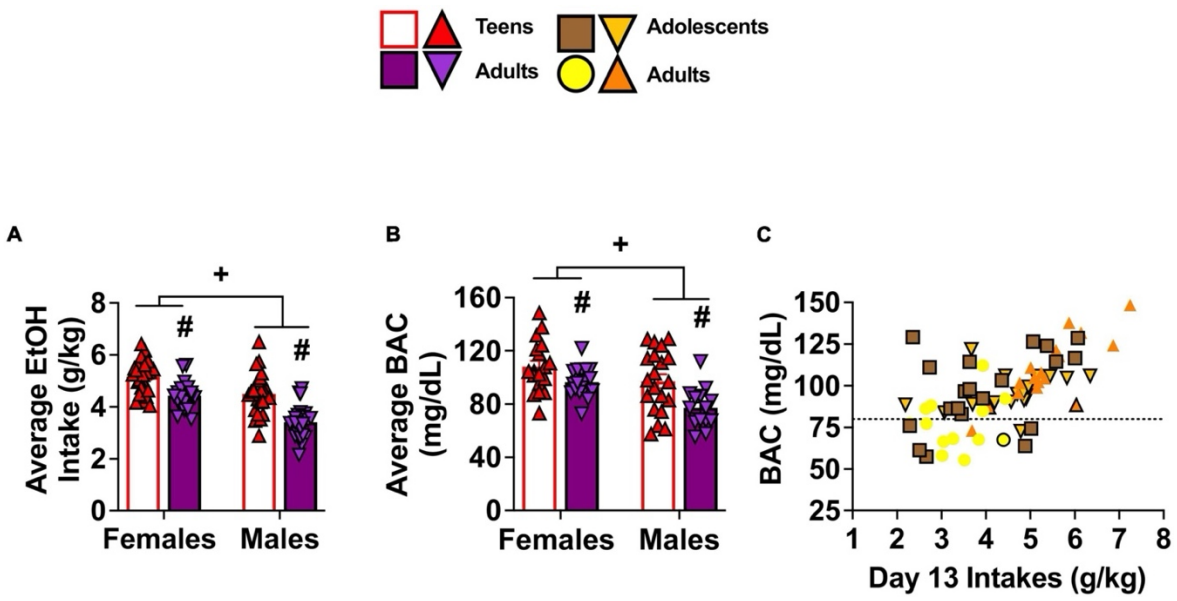


Figure 4.2. Depiction of age and sex differences in alcohol intake and corresponding BACs. As there were no Withdrawal effects or interactions, the data for alcohol intake and BACs are collapsed across mice slated to be tested on WD1 and WD30. **(A)** On average, adolescent (Adol.) mice consumed more alcohol than adult mice and females consumed more than males [females: adolescents (n = 24), adults (n = 20); males: adolescents (n = 24), adults (n = 24)]. **(B)** The average BAC levels obtained on Day 13 of drinking paralleled group differences in alcohol intake [females: adolescents (n = 18), adults (n = 18); males: adolescents (n = 20), adults (n = 14)] and **(C)** a positive correlation was observed between BACs and alcohol consumption on Day 13 of drinking [sample sizes same as panel **(B)**]. The data in panels **(A,B)** are presented as the means ± SEMs for the respective number of mice indicated above. $^{+}p < 0.05$, Female vs. Male (main Sex effect); $^{\#}p < 0.05$, adolescents vs. adults (main Age effect).

4.3.2. Light Dark Box Shuttle Test

4.3.2.1. Latency to First Enter Light Side

A Sex × Age × Drinking History ANOVA failed to detect any significant differences for the latency to first enter the light-side of the light-dark shuttle-box on either WD1 (**Figure 4.3A**) (3-way ANOVA: $p = 0.883$, $\eta 2p = 0.000$; all other p's > 0.160) or WD30 (**Figure 4.3B**; 3-way ANOVA: $p = 0.330$, $\eta 2p = 0.011$, all other p's > 0.228).

4.3.2.2. Time in the Light Side

On WD1, an Age × Drinking History interaction [$F(1,85) = 6.65$, $p = 0.012$, $\eta 2p = 0.073$] and a Sex × Age interaction [$F(1,85) = 4.35$, $p = 0.040$, $\eta 2p = 0.049$] were found for the time spent in the light side (**Figure 4.3C**). As illustrated in **Figure 4.3D**, the Age × Drinking History interaction reflected less time spent in the light-side by adult binge-drinking mice versus both adult water controls ($p = 0.069$, $d = 0.554$) and adolescent binge-drinking mice ($p = 0.004$, $d = 0.935$). Adolescent water control mice also spent less time in the light side when compared to their binge-drinking counterparts ($p = 0.075$, $d = 0.532$). The Sex × Age interaction (**Figure 4.3E**) reflected more time spent in the light-side by adult female versus adult male mice ($p = 0.001$, $d = 1.001$), with no sex difference apparent in adolescent animals ($p = 0.680$, $d = 0.122$). Additionally, adolescent males spent more time in the light-side compared to the adult males ($p = 0.006$, $d = 0.832$). On WD30, no significant effects or interactions were detected (**Figure 4.3F**; 3-way ANOVA: $p = 0.396$, $\eta 2p = 0.009$; all other p's > 0.140).

4.3.2.3. Light Side Entries

On WD1, a Sex × Age × Drinking History ANOVA detected a significant Sex × Drinking History [$F(1,85) = 3.59$, $p = 0.062$, $\eta 2p = 0.041$] and an Age × Drinking History interaction [$F(1,85) = 4.75$, $p = 0.032$, $\eta 2p = 0.053$] for the number of entries into the light-side (**Figure 4.3G**). As illustrated in **Figure 4.3H**, the Sex × Drinking History interaction reflected a higher number of light side entries in male binge-drinking mice versus the female binge-drinking mice ($p = 0.072$, $d = 0.563$). Although inspection of **Figure 4.3I** suggested that adolescent binge-drinking mice made more light side entries than their water controls,

while the opposite was true for adult binge-drinking mice, deconstruction of the Age × Drinking History interaction indicated no significant Water- EtOH difference in the adolescent or adult mice (Adolescents: $p = 0.119$, $d = 0.459$; Adults: $p = 0.135$, $d = 0.456$). On WD30, a main Sex effect was observed for the number of light-side entries [$F(1,88) = 9.48$, $p = 0.003$, $\eta 2p = 0.097$; all other p's > 0.203], with females entering the light-side more, overall, than males (**Figure 4.3J**).

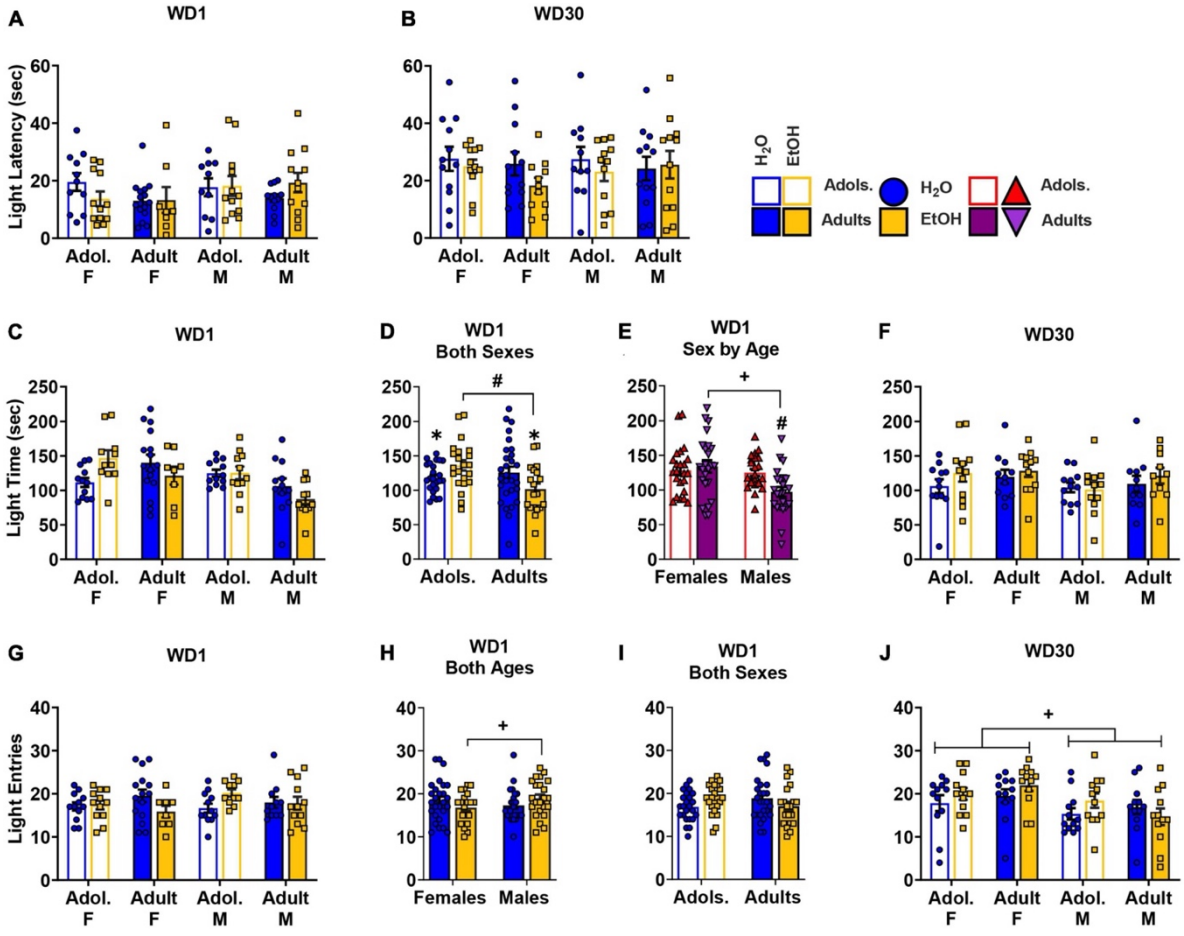


Figure 4.3. Depiction of the results of the Sex × Age × Drinking History ANOVAs conducted for behavior in the light dark box shuttle test. No group differences were observed for the latency to enter the light side of the shuttle box on either WD1 **(A)** [females: adolescents/Adol. H2O ($n = 11$), EtOH ($n = 12$); adults H2O ($n = 16$), EtOH ($n = 8$); males: adolescent H2O ($n = 10$), EtOH ($n = 12$); adults H2O ($n = 12$), EtOH ($n = 12$)] or

WD30 **(B)** [females: adolescents H2O (n = 12), EtOH (n = 12); adults H2O (n = 12), EtOH (n = 11); males: adolescent H2O (n = 11), EtOH (n = 12); adults H2O (n = 12), EtOH (n = 12)]. **(C)** Summary of the results for the time spent in the light side for all groups tested on WD1 [females: adolescents H2O (n = 12), EtOH (n = 11); adults H2O (n = 16), EtOH (n = 8); males: adolescent H2O (n = 12), EtOH (n = 11); adults H2O (n = 12), EtOH (n = 11)]. **(D)** On WD1, an Age by Drinking History interaction was observed for the time spent in the light side that reflected less time spent by adult binge-drinking (EtOH) mice versus both adult water (H2O) and adolescent (Adol.) EtOH mice. Additionally, adolescent H2O mice spent less time in the light side than their age- matched EtOH counterparts [adolescents: H2O (n = 24), EtOH (n = 22); adults: H2O (n = 28), EtOH (n = 19)]. **(E)** Also on WD1, we detected a Sex by Age interaction that reflected more time spent on the light side by adult females (F) versus adult males (M), while no sex difference was apparent in adolescent mice. Adolescent males, however, spent more time in the light side compared to the adult males [females: adolescents (n = 23), adults (n = 24); males: adolescents (n = 23), adults (n = 23)]. **(F)** On WD30, no group differences were detected for the total time spent in the light side of the shuttle box [females: adolescents H2O (n = 12), EtOH (n = 12); adults H2O (n = 11), EtOH (n = 12); males: adolescent H2O (n = 12), EtOH (n = 12); adults H2O (n = 11), EtOH (n = 10). **(G)** Results for the total number of entries into the light side of the shuttle box test indicated significant interactions on WD1 between Sex by Drinking History and Age by Drinking History [females: adolescents H2O (n = 12), EtOH (n = 12); adults H2O (n = 15), EtOH (n = 8); males: adolescent H2O (n = 12), EtOH (n = 11); adults H2O (n = 11), EtOH (n = 12)]. **(H)** Follow-up analysis of the Sex by Drinking History interaction revealed that male EtOH mice exhibited more entries into the light side compared to female EtOH mice [females: H2O (n = 27), EtOH (n = 20); males: H2O (n = 23), EtOH (n = 23)]. **(I)** The Age by Drinking History interaction on WD1 did not reflect any significant effect of EtOH in either age group [adolescents: H2O (n = 24), EtOH (n = 23); adults: H2O (n = 26), EtOH (n = 20)]. **(J)** On WD30, female mice exhibited a greater number of entries into the light side compared to male mice, irrespective of age or drinking condition [females: adolescents H2O (n = 1)2, EtOH (n = 12); adults H2O (n = 12), EtOH (n = 12); males: adolescent H2O (n = 12), EtOH (n = 12); adults H2O (n = 12), EtOH (n = 12). The data represent the means ± SEMs for the number of mice indicated above. $^{+}p < 0.10$, Female vs. Male; $^{\#}p < 0.10$, adolescents vs. adults.

4.3.3. Marble Burying Test

The data for the number of marbles buried on WD1 by all of the groups are presented in **Figure 4.4A**. An analysis of these data indicated more marbles buried by adult versus adolescent mice (**Figure 4.4B**) [Age effect: $F(1,88) = 4.01$, $p = 0.048$, $\eta 2p = 0.044$], but no other effects or interactions were found at this withdrawal time-point (Sex × Age × Drinking History ANOVA: $p = 0.511$, $\eta 2p = 0.005$; all other p's > 0.496). The data for the number of

marbles buried on WD30 by all of the groups are presented in **Figure 4.4C**. For these mice, no significant interactions were found [3-way ANOVA, $p = 0.104$, $\eta 2p = 0.030$; all other interactions p's > 0.255]. However, significant main effects of Sex (**Figure 4.4C**) and Age (**Figure 4.4D**) were detected [Sex effect: $F(1,88) = 10.16$, $p = 0.002$, $\eta 2p = 0.104$]; Age effect: $F(1,88) = 3.77$, $p = 0.055$, $\eta 2p = 0.41$], indicating that females buried more marbles versus the male mice, and adult mice buried more marbles compared to their adolescent counterparts.

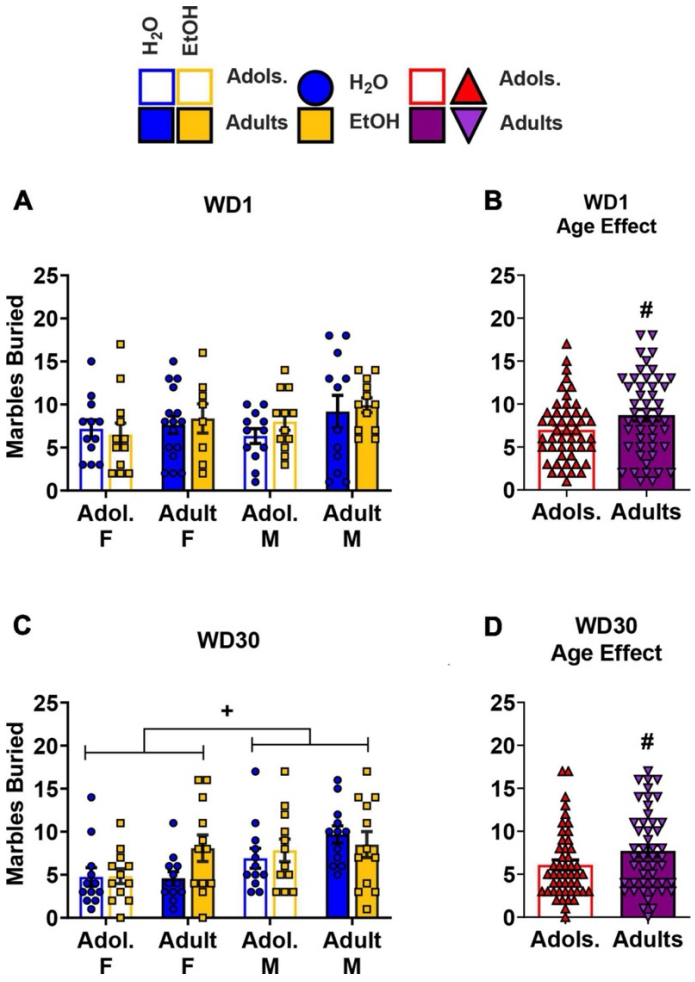


Figure 4.4. Depiction of the results of the Sex × Age × Drinking History ANOVAs conducted for behavior in the marble burying test. (A) On WD1, no significant interactions were observed for the number of marbles buried [females: adolescents/Adol. H2O ($n = 12$), EtOH ($n = 12$); adults H2O ($n = 16$), EtOH ($n = 8$); males: adolescent H2O

(*n* = 12), EtOH (*n* = 12); adults H2O (*n* = 12), EtOH (*n* = 12)]. However, **(B)** adult mice buried more marbles than the adolescent mice [adolescents (*n* = 48), adults (*n* = 48)]. **(C)** On WD30, females buried a greater number of marbles compared to the male mice [females: adolescents H2O (*n* = 12), EtOH (*n* = 12); adults H2O (*n* = 12), EtOH (*n* = 12); males: adolescent H2O (*n* = 12), EtOH (*n* = 12); adults H2O (*n* = 12), EtOH (*n* = 12)]. **(D)** Similar to the mice in WD1, adult mice buried more marbles than their adolescent counterparts [adolescents (*n* = 48), adults (*n* = 48)]. The data represent the means ± SEMs for the number of mice indicated above. $^{+}p < 0.10$, Female vs. Male; $^{\#}p < 0.10$, adolescents vs. adults.

4.3.4. Porsolt Forced Swim Test

4.3.4.1. Latency to First Immobile Episode

The data for the latency to first float in the forced swim test on WD1 are presented in **Figure 4.5A**. A Sex × Age × Drinking History ANOVA detected no interactions with respect to the latency to first float in the forced swim test on WD 1 [Sex × Age Drinking History ANOVA: $p = 0.161$, $\eta 2p = 0.024$, all other interactions p's > 0.525]. However, a significant main effect of Drinking History was detected (**Figure 4.5B**) [$F(1,80) = 4.34$, $p = 0.040$, $\eta 2p = 0.051$] that reflected a longer latency to immobility in binge-drinking mice, relative to their water-drinking counterparts. For the mice tested on WD30, a significant main effect of Sex [$F(1,84) = 8.07$, $p = 0.006$, $\eta 2p = 0.088$] reflected a shorter immobile latency for females versus males, irrespective of their binge-drinking history or age of binge-drinking onset (**Figure 4.5C**; all other *p's* > 0.102).

4.3.4.2. Time Spent Immobile

The data for the time spent immobile during the forced swim test on WD1 are presented in **Figure 4.5D**. On WD1, a significant Sex x Age x Drinking History interaction was observed for the total time spent immobile during the forced swim test [$F(1,84) = 4.08$, $p = 0.047$, $\eta 2p = 0.046$]. To investigate potential age differences, this interaction was split

along the Sex factor and revealed a significant Age x Drinking History interaction for the male mice (**Figure 4.5D**, right) [$F(1,43) = 4.41$, $p = 0.042$, $\eta 2p = 0.093$], but no significant main effect or interactions for the females (**Figure 4.5D**, left) [ANOVA: $p = 0.378$, $\eta 2p = 0.019$]. As illustrated in **Figure 4.5E**, adolescent male binge-drinking mice spent more time immobile than their water-drinking counterparts ($p = 0.031$, $d = 0.032$) and the adult male binge-drinking mice ($p = 0.004$, $d = 1.260$). To analyze for sex-related differences in the time spent immobile, the 3-way interaction was also deconstructed along the Age variable. This deconstruction found a Sex × Drinking History interaction for the adolescent mice, but not for the adult mice [Adolescent: $F(1,42) = 4.08$, $p = 0.050$, $\eta 2p = 0.089$; Adult ANOVA: $p = 0.419$, $\eta 2p = 0.016$]. As illustrated in **Figure 4.5F** (left vs. right), adolescent female water-drinking mice spent more time immobile than their male counterparts ($p = 0.050$, $d = 0.844$). Additionally, the adolescent male binge-drinking mice also spent more time immobile than the water-drinking control mice (**Figure 4.5F**, right; $p = 0.055$, $d = 0.823$).

The data for the time spent immobile on WD30 is presented in **Figure 4.5G**. On WD30, a Sex × Drinking History interaction was found for the total time spent immobile [$F(1,87) = 3.33$, $p = 0.072$, $\eta 2p = 0.037$]. This interaction reflected a longer time spent immobile by female binge-drinking mice compared to the male binge-drinking mice (**Figure 4.5H**; $p = 0.001$, $d = 0.991$). No other significant interactions were observed for this variable on WD30 [3-way ANOVA: $p = 0.641$, $\eta 2p = 0.003$; all other p's > 0.132).

4.3.4.3. Immobile Episodes

The data for the number of immobile episodes on WD1 are presented in **Figure 4.5I**. A 3-way Sex × Age × Drinking History interaction was revealed for this variable [$F(1,88) = 8.29$, $p = 0.005$, $\eta 2p = 0.086$]. To examine for age differences, the interaction was first deconstructed along the Sex factor, which resulted in significant Age × Drinking History interactions for both male [$F(1,44) = 5.05$, $p = 0.030$, $\eta 2p = 0.103$] and female subjects [$F(1,44) = 3.39$, $p = 0.072$, $\eta 2p = 0.072$]. As illustrated for males in **Figure 4.5I** (right), the 2-way interaction reflected a higher number of immobile episodes for the adolescent binge-drinking mice versus their water-drinking counterparts ($p = 0.082$, $d = 0.727$). Additionally, adolescent male binge-drinking mice had a higher number of immobile episodes versus adult binge-drinking males ($p = 0.006$, $d = 1.81$). In contrast, as illustrated in **Figure 4.5I** (left), the 2-way interaction detected in females reflected water- alcohol differences for adult mice only ($p = 0.056$, $d = 0.849$). We also observed a higher number of immobile episodes for adolescent water-drinking females versus their adult counterparts ($p = 0.021$, $d = 0.911$). To examine for sex-related differences in basal and withdrawal-induced behavior, the 3-way interaction was analyzed also along the Age factor. This deconstruction revealed a significant Sex × Drinking History interaction for both adult [$F(1,44) = 5.603$, $p = 0.022$, $\eta 2p = 0.113$] and adolescent mice [$F(1,44) = 2.841$, $p = 0.099$, $\eta 2p = 0.061$]. Thus, the data in **Figure 4.5I** was re-arranged to better illustrate the age-dependency of these sex differences (**Figure 4.5J**). As illustrated in **Figure 4.5J** (right), the Sex × Drinking History interaction in adult mice reflected a sex difference in binge- drinkers, but not water controls, where adult female binge-drinkers had more immobile episodes versus the adult male binge-drinking mice ($p = 0.028$, $d = 1.035$). For the adolescent mice (**Figure 4.5J**, left), no significant water-alcohol

differences were observed in female mice, however, adolescent male binge-drinking mice had more immobile episodes than their water-drinking counterparts ($p = 0.086$, $d = 0.717$).

The data for the number of immobile episodes on WD30 are presented in **Figure 4.5K**. On WD30, significant Sex × Drinking History [$F(1,87) = 3.88$, $p = 0.052$, $\eta 2p = 0.043$] and Age x Drinking History [$F(1,87) = 2.87$, $p = 0.094$, $\eta 2p = 0.032$] interactions were detected. As illustrated in **Figure 4.5L**, male binge- drinking mice exhibited fewer immobile episodes than their water controls ($p = 0.022$, $d = 0.674$), while female binge-drinking mice exhibited more immobile episodes than their male binge- drinking counterparts ($p = 0.001$, $d = 0.973$). As illustrated in **Figure 4.5M**, the Age x Drinking History interaction revealed fewer immobile episodes by the adolescent binge-drinking mice versus their water controls ($p = 0.037$, $d = 0.618$) and the adolescent water control mice also exhibited more immobile episodes than their adult counterparts ($p = 0.051$, $d = 0.572$). No other significant interactions were observed (3-way ANOVA: $p = 0.773$, $\eta 2p = 0.001$).

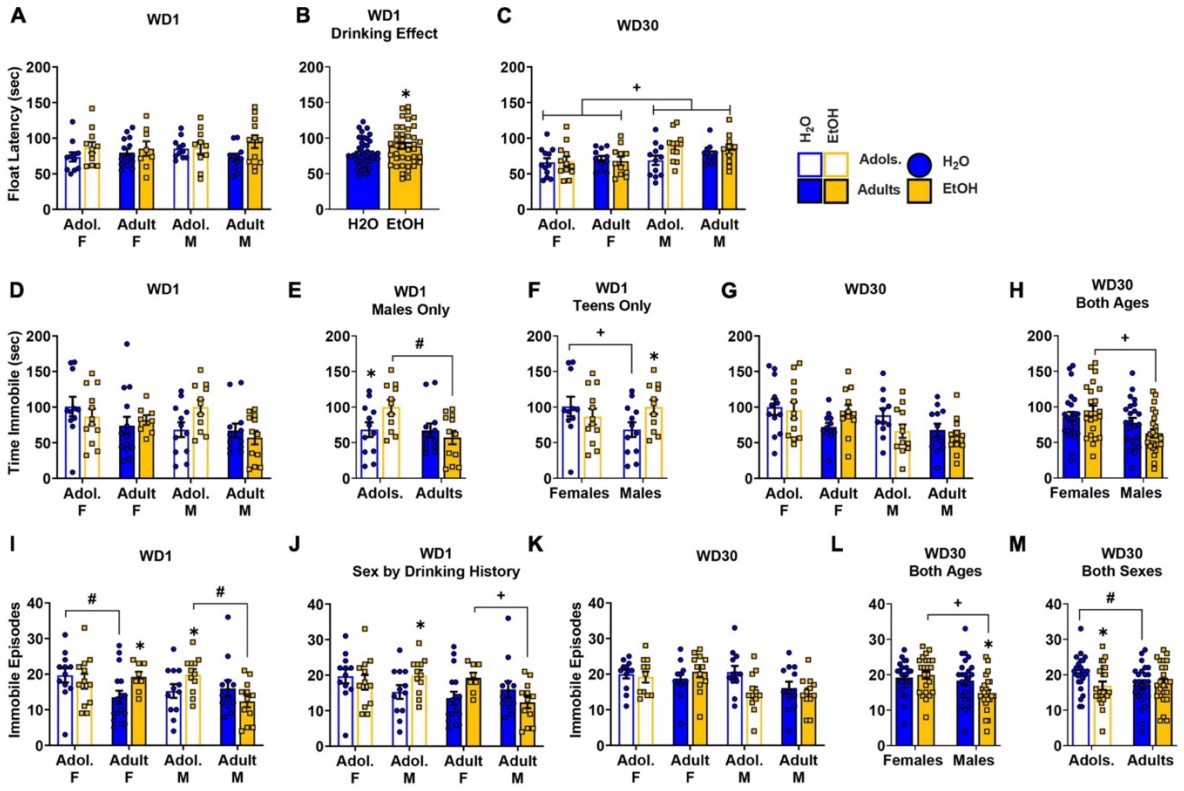


Figure 4.5. Depiction of the results of the Sex × Age × Drinking History ANOVAs conducted for behavior in the Porsolt forced swim test. (A) On WD1, we detected no significant 3-way interaction for the latency to immobility [females: adolescents/Adol. H2O (*n* = 11), EtOH (*n* = 11); adults H2O (*n* = 14), EtOH (*n* = 8); males: adolescent H2O (*n* = 11), EtOH (*n* = 10); adults H2O (*n* = 11), EtOH (*n* = 12)]. **(B)** However, binge-drinking (EtOH) mice had a longer latency to immobility, overall, than water (H2O) mice, on WD1 [sample size. **(C)** Overall, males (M) exhibited a longer latency to immobility on WD30 than females (F) [females: adolescents H2O (*n* = 12), EtOH (*n* = 12); adults H2O (*n* = 11), EtOH (*n* = 11); males: adolescent H2O (*n* = 12), EtOH (*n* = 12); adults H2O (*n* = 12), EtOH (*n* = 10)]. **(D)** On WD1, a significant 3-way interaction was detected for the time spent immobile, that reflected a longer time spent immobile by adolescent (Adol.) EtOH males versus both adolescent H2O and adult EtOH males **(E)** [females: adolescents H2O (*n* = 11), EtOH (*n* = 12); adults H2O (*n* = 14), EtOH (*n* = 8); males: adolescent H2O (*n* = 12), EtOH (*n* = 11); adults H2O (*n* = 12), EtOH (*n* = 12)]. **(F)** When deconstructed along the Age factor, adolescent male EtOH mice spent more time immobile than their H2O counterparts [females: H2O (*n* = 11), EtOH (*n* = 12); males: H2O (*n* = 12), EtOH (*n* = 11). **(G)** For WD30, no significant 3-way interaction was detected for the time spent immobile [females: adolescents H2O (*n* = 12), EtOH (*n* = 12); adults H2O (*n* = 12), EtOH (*n* = 12); males: adolescent H2O (*n* = 11), EtOH (*n* = 12); adults H2O (*n* = 12), EtOH (*n* = 12)]. **(H)** However, a Sex by Drinking History interaction found that female EtOH mice spent more time immobile than male EtOH mice [females: H2O (*n* = 24), EtOH (*n* = 24); males: H2O (*n* = 23), EtOH (*n* = 24)]. **(I)** A significant Sex by Age by Drinking History interaction was observed for the

number of immobile episodes on WD1, and results deconstructed along the Sex factor revealed that adolescent male EtOH mice had more immobile episodes than their H2O counterparts and adult EtOH males, while adult female EtOH mice also had more episodes than their H2O counterparts [females: adolescents H2O (n = 12), EtOH (n = 12); adults H2O (n = 16), EtOH (n = 8); males: adolescent H2O (n = 12), EtOH (n = 12); adults H2O (n = 12), EtOH (n = 12)]. **(J)** Analysis along the Age factor identified sex-related differences where adult female EtOH had more immobile episodes than adult male EtOH, and adolescent male EtOH had more immobile episodes than their H2O counterparts [sample sizes same as panel **(J)**]. **(K)** For WD30, a significant Sex by Drinking History and Age by Drinking History interaction were detected [females: adolescents H2O (n = 12), EtOH (n = 11); adults H2O (n = 12), EtOH (n = 12); males: adolescent H2O (n = 12), EtOH (n = 12); adults H2O (n = 12), EtOH (n = 12)]. **(L)** Follow-up analyses revealed that male H2O mice had more immobile episodes than their EtOH counterparts and that females EtOH mice had more immobile episodes than their EtOH male counterparts [females: H2O (n = 24), EtOH (n = 23); males: H2O (n = 24), EtOH (n = 24)]. **(M)** An Age by Drinking History interaction indicated that adolescent H2O mice had more immobile episodes than their EtOH counterparts and the adult H2O mice [adolescents: H2O (n = 24), EtOH (n = 23); adults: H2O (n = 24), EtOH (n = 24)]. The data represent the means ± SEMs for the number of mice indicated above. *$p < 0.10$, EtOH vs. H2O; $^{+}p < 0.10$, Female vs. Male; $^{\#}p < 0.10$, adolescents vs. adults.

4.3.5. Morris Water Maze

4.3.5.1. Flag Test

Sex × Age × Drinking History ANOVAs failed to detect any significant interactions or main effects for the time taken to locate the flagged platform during either early [all p's > 0.582] or later withdrawal [all p's > 0.343]. The data are presented in **Table 4.4.** and indicate comparable visual and swimming ability across our different experimental groups prior to maze training. These findings also indicate that group differences in the Porsolt swim test, conducted 1–2 days prior (**Figure 4.5**), did not carry over to the Morris water maze.

	Early withdrawal		Late withdrawal	
	Females	Males	Females	Males
Adolescent-H2O	70.39 ± 13.60 *n* = 12	70.32 ± 12.16 *n* = 12	53.35 ± 13.20 *n* = 12	46. 62 ± 11.39 *n* = 12
Adolescent-EtOH	67.89 ± 13.18 *n* = 12	57.23 ± 12.35 *n* = 12	54.31 ± 13.32 *n* = 12	59.58 ± 13.12 *n* = 12
Adult-H2O	74.84 ± 10.89 *n* = 16	63. 76 ± 12.52 *n* = 12	53.37 ± 13.48 *n* = 12	69.49 ± 14.17 *n* = 12
Adult-EtOH	68.45 ± 12.41 *n* = 8	65.75 ± 13.17 *n* = 12	38.28 ± 9.50 *n* = 12	54.65 ± 11.98 *n* = 12

The data represent the means ± SEMs for the number of mice indicated.

Table 4.4. Summary of the negative results for the time taken (in sec) to locate the flagged platform in the Morris water maze.

4.3.5.2. Morris Maze Acquisition

No significant Day × Sex × Age × Drinking History interaction was noted for the time taken to locate the hidden platform across the 4 days of the Morris maze acquisition for the mice tested in early withdrawal (4-way ANOVA: $p = 0.865$, $\eta p2 = 0.001$). As depicted in **Figures 4.6A–D**, all mice successfully acquired the maze as indicated by a main Day effect [$F(1.49, 123.79) = 65.95$, $p < 0.001$, $\eta 2p = 0.443$; all other p's > 0.118]. We also detected no significant Day × Sex × Age × Drinking History interaction for the time taken to complete the Morris maze by mice tested in later withdrawal [**Figures 4.6E–H**; 4-way ANOVA: $p = 0.464$, $\eta 2p = 0.008$]. However, a significant Day × Age interaction was detected in later withdrawal [$F(1.41, 116.64) = 5.83$, $p = 0.009$, $\eta 2p = 0.066$]. As illustrated in **Figure 4.6I**, this interaction reflected more time taken by adolescent-onset versus adult-onset mice to locate the hidden platform on the first day of training, irrespective of their sex or alcohol-drinking history ($p = 0.004$).

4.3.5.3. Probe Test

The data for the latency to enter the platform's former location on WD1 are presented in **Figure 4.6J**. Analyses of a Sex × Age × Drinking History ANOVA for the mice tested in early withdrawal failed to detect a significant 3-way interaction for the latency to first enter the platform's former location ($p = 0.333$, $\eta 2p = 0.011$); however, a Age x Drinking History interaction was detected for this variable [$F(1,85) = 5.50$, $p = 0.021$, $\eta 2p = 0.061$]. This interaction reflected a shorter latency to first enter the platform location by adolescent-onset mice relative to their age- matched water-drinking counterparts ($p = 0.047$, $d = 0.589$), and to the adult binge-drinking mice (**Figure 4.6K**; $p = 0.036$, $d = 0.651$). For the mice tested in later withdrawal, no significant main effects or interactions were found with respect to this variable [**Figure 4.6L**; Sex × Age × Drinking History ANOVA: $p = 0.703$, $\eta 2p = 0.002$, all other p's > 0.306].

As alternate indices of spatial recall, we also examined the number of entries into the platform's former location. No significant main effects or interactions were observed for the number of entries into the platform's former location for mice tested in early withdrawal [**Figure 4.6M**; 3-way ANOVA: $p = 0.444$, $\eta 2p = 0.007$; all other p's > 0.386]. However, a significant Age x Drinking History interaction was observed for the number of former platform entries for the mice tested in later withdrawal [**Figure 4.6N**; $F(1,87) = 6.63$, $p = 0.012$, $\eta 2p = 0.071$]. This interaction reflected a trend for more entries by adolescent-onset water controls versus their binge-drinking counterparts, with a medium effect size (**Figures 4.6O, F**, left; $p = 0.087$, $d = 0.500$), with a similarly sized, but opposite, group difference was noted for the adult-onset mice (**Figure 4.6O**, right; $p = 0.060$, $d = 0.557$). Lastly, adolescent-

onset water-drinking controls made more entries, overall, than their adult-onset counterparts (**Figure 4.6O**; $p = 0.036$, $d = 0.614$).

4.3.5.4. Reversal Test

For the mice tested in early withdrawal (**Figures 4.6P–S**), a Trial × Sex × Age × Drinking History ANOVA revealed no significant group differences for the time taken to locate the repositioned platform during the reversal test [all ANOVAs *p's* > 0.158]. In contrast, a significant Trial x Age interaction was detected for the mice tested in later withdrawal (**Figures 4.6T–W**) [$F(1.88, 152.49) = 5.66$, $p = 0.001$, $\eta 2p = 0.065$] that reflected a longer time taken to find the repositioned platform by adult-onset versus adolescent-onset mice on the initial reversal trial (**Figure 4.6X**; Trial 1: $p = 0.034$). No other significant interactions were observed between the binge-drinking and water-drinking groups, however, a main effect of Trial illustrated a progressive reduction in the time required to locate the platform [Trial Effect: $F(1.88,152.49) = 46.07$, $p < 0.001$, $\eta 2p = 0.363$].

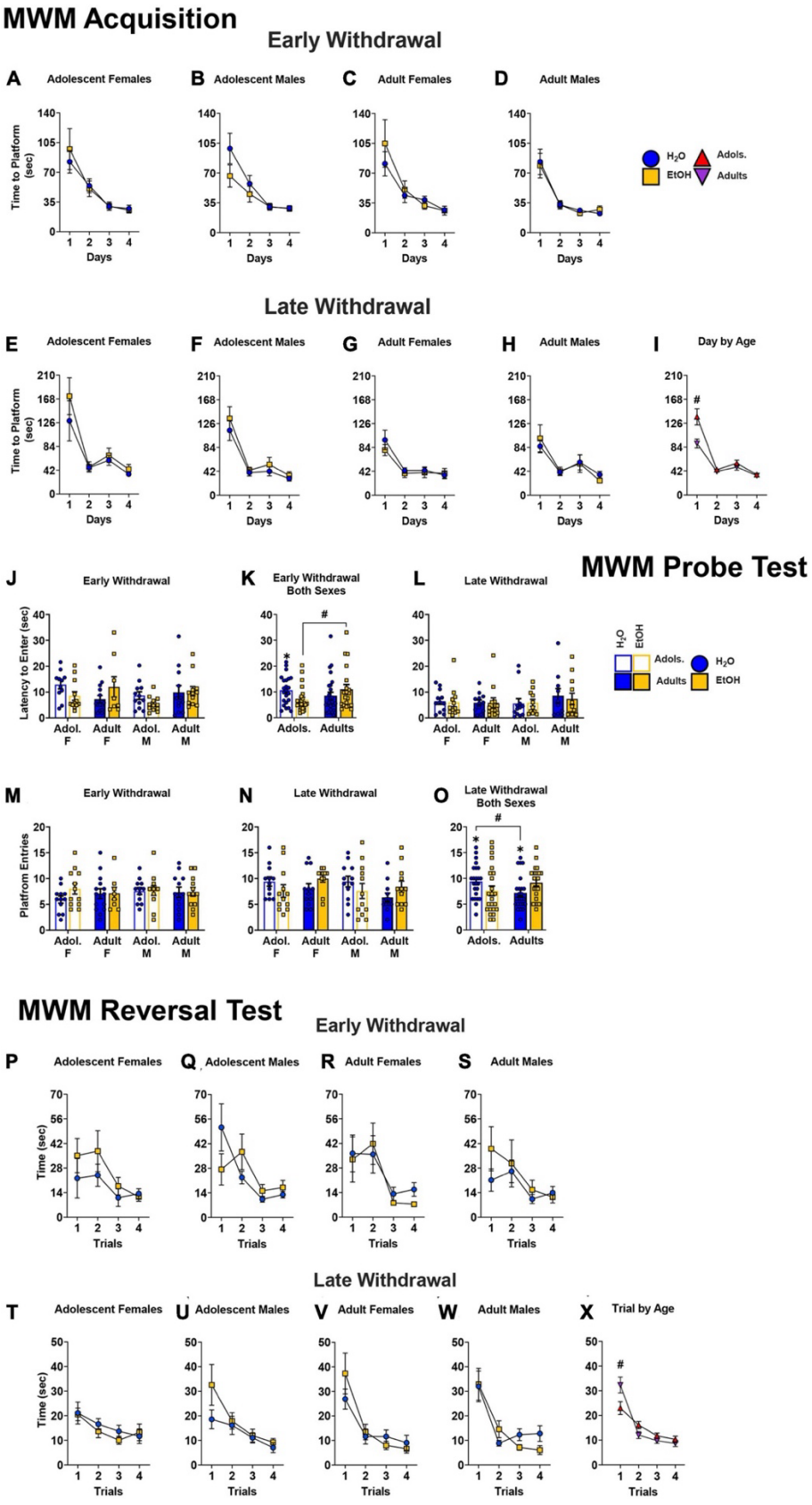

MWM Acquisition
Early Withdrawal
A Adolescent Females
B Adolescent Males
C Adult Females
D Adult Males
Time to Platform (sec)
Days
H2O
EtOH
Adols.
Adults
Late Withdrawal
E Adolescent Females
F Adolescent Males
G Adult Females
H Adult Males
I Day by Age
MWM Probe Test
J Early Withdrawal
K Early Withdrawal Both Sexes
L Late Withdrawal
Latency to Enter (sec)
Adol. F
Adult F
Adol. M
Adult M
Adols.
Adults
M Early Withdrawal
N Late Withdrawal
O Late Withdrawal Both Sexes
Platfrom Entries
MWM Reversal Test
Early Withdrawal
P Adolescent Females
Q Adolescent Males
R Adult Females
S Adult Males
Time (sec)
Trials
Late Withdrawal
T Adolescent Females
U Adolescent Males
V Adult Females
W Adult Males
X Trial by Age

Figure 4.6. Depiction of the results of the Day × Sex × Age × Drinking History mixed-model ANOVAs evaluating spatial learning during the different phases of testing in the Morris water maze. **(A–D)** No group differences were noted for the average time taken by mice tested in early alcohol withdrawal to locate the hidden platform during Morris maze acquisition. The sample sizes for mice tested on WD1 are the following: **(A)** H2O (*n* = 9), EtOH (*n* = 12); **(B)** H2O (*n* = 11), EtOH (*n* = 12); **(C)** H2O (*n* = 15), EtOH (*n* = 8); **(D)** H2O (*n* = 12), EtOH (*n* = 12) **(E–H)** For mice trained during later withdrawal, we detected no significant Day by Sex by Age by Drinking History interaction. The sample sizes for mice tested on WD are the following); **(E)** H2O (*n* = 12), EtOH (*n* = 11); **(F)** H2O (*n* = 12), EtOH (*n* = 9); **(G)** H2O (*n* = 12), EtOH (*n* = 12); **(H)** H2O (*n* = 12), EtOH (*n* = 11). **(I)** However, a significant Day by Age interaction was detected that reflected a longer time taken by adolescent versus adult mice to locate the platform on the first day training [adolescents (*n* = 44), adults (*n* = 47)]. **(J)** When tested in early alcohol withdrawal, no significant 3-way interaction was detected for the latency to enter the platform's former location [females: adolescent H2O (*n* = 9); adolescent EtOH (*n* = 12); adult H2O (*n* = 11); adult EtOH (*n* = 12); males: adolescent H2O (*n* = 15); adolescent EtOH (*n* = 8); adult H2O (*n* = 12); adult EtOH (*n* = 12)]. **(K)** On the probe test, an Age by Drinking History interaction indicated that adolescent-onset mice exhibited a shorter latency to enter the platform's former location in the NE quadrant compared to their age-matched water control counterparts and the adult-onset mice [adolescents: H2O (*n* = 24); EtOH (*n* = 20); adults: H2O (*n* = 23); EtOH (*n* = 24). **(L)** No significant group differences were found for this measure in mice tested in later withdrawal [females: adolescent H2O (*n* = 12); adolescent EtOH (*n* = 11); adult H2O (*n* = 12); adult EtOH (*n* = 19); males: adolescent H2O (*n* = 12); adolescent EtOH (*n* = 12); adult H2O (*n* = 12); adult EtOH (*n* = 12)]. **(M)** We also did not detect group differences on WD1 with regards of the number of entries to the former site of the platform [samples sizes same as panel **(A)**]. **(N)** However, on WD30, a significant Age by Drinking History interaction was observed [sample sizes same as Panel **(C)**]. **(O)** This interaction reflected trends for more entries by adolescent EtOH versus adolescent H2O mice, as well as fewer entries by adult EtOH versus adult H2O mice. Additionally, adolescent H2O mice made significantly more entries than adult H2O mice [adolescents (*n* = 44), adults (*n* = 47)]. For the data from the reversal learning phase of the study, Trial by Sex by Age by Drinking History ANOVAs revealed no significant group differences for the time taken to locate the repositioned platform during the reversal test when mice were tested in either early **(P–S)** or late withdrawal **(T–W)**. Sample sizes are the following: **(P)** H2O (*n* = 10), EtOH (*n* = 12); **(Q)** H2O (*n* = 12), EtOH (*n* = 12); **(R)** H2O (*n* = 15), EtOH (*n* = 8); **(S)** H2O (*n* = 11), EtOH (*n* = 11); **(T)** H2O (*n* = 12), EtOH (*n* = 10); **(U)** H2O (*n* = 11), EtOH (*n* = 10); **(V)** H2O (*n* = 11), EtOH (*n* = 12); **(W)** H2O (*n* = 12), EtOH (*n* = 11). **(X)** However, a significant Trial by Age interaction was observed for the mice tested in late withdrawal that reflected a longer latency of adult-onset versus adolescent-onset mice to locate the repositioned platform on the first reversal trial [adolescents (*n* = 43), adults (*n* = 46)]. The data represent the means ± SEMs for the number of mice indicated above. $^{\#}p < 0.05$, adolescents vs. adults.

4.3.6. Radial Arm Water Maze

4.3.6.1. Number of Reference Memory Errors

For the mice tested in early withdrawal, a significant Day × Sex × Age × Drinking History interaction was detected for the number of reference memory errors during the first week of radial arm maze training (**Figures 4.7A–D**) [$F(4.32, 380.37) = 3.27$, $p = 0.010$, $\eta 2p = 0.036$]. This 4-way interaction was first analyzed along the Sex factor and indicated a significant Day × Age × Drinking History interaction for the female mice [$F(4.20, 184.67) = 4.00$, $p = 0.003$, $\eta 2p = 0.083$]. The Day x Age x Drinking History interaction observed in female mice was further deconstructed along the Age factor and indicated a significant Day × Drinking History interaction for the adolescent-onset females [$F(3.93, 86.46) = 3.03$, $p = 0.022$, $\eta 2p = 0.121$]. However, while it appeared that adolescent- onset binge-drinking females committed more reference memory errors than their water-drinking counterparts on several days during this initial training (**Figure 4.7A**), *post-hoc* tests did not indicate any statistically significant water-alcohol differences (all p's > 0.072). The comparable follow-up analysis of the significant Day × Age × Drinking History interaction for adult-onset females indicated only a significant main effect of Day (**Figure 4.7C**) [$F(3.69,127.17) = 3.76$, $p = 0.009$, $\eta 2p = 0.146$]. Thus, a prior history of binge-drinking during adulthood did not influence reference memory in adult females tested during early alcohol withdrawal. For the males tested in early withdrawal, no significant Day × Age × Drinking History interaction was found upon deconstruction of the significant 4-way interaction along the Age factor [ANOVA: $p = 0.524$, $\eta 2p = 0.019$]. However, a Day x Age interaction was observed [$F(4.00, 175.76) = 2.18$, $p = 0.074$, $\eta 2p = 0.047$], that reflected a trend toward more reference

memory errors committed by adult versus adolescent males on day 6 of training only (**Figures 4.7B, D**; $p = 0.061$).

For mice tested in later withdrawal (**Figures 4.7E–H**), a significant Day × Age interaction [$F(5, 440) = 2.72$, $p = 0.020$, $\eta 2p = 0.030$] was detected. However, *post hoc* analyses indicated that this interaction reflected more reference memory errors committed by adults vs. adolescents only on day 4 of training ($p = 0.041$) and thus, this interaction is not depicted.

4.3.6.2. Working Memory Correct Errors

Analyses of the data from the mice tested in early withdrawal identified a significant Day × Sex × Age × Drinking History interaction for the number of working memory correct errors during the first week of testing (**Figures 4.7I– L**) [$F(4.48,394.13) = 2.43$, $p = 0.041$, $\eta 2p = 0.027$]. While deconstruction along the Sex factor indicated no significant interactions [ANOVA for females, p's > 0.212; ANOVA for males, p's > 0.162], deconstruction along the Age factor revealed a significant Day x Sex interaction for the adolescent-onset mice [$F(4.31,189.61) = 2.76$, $p = 0.026$, $\eta 2p = 0.059$], that reflected a greater number working memory correct errors in males versus females only on day 3 of radial arm maze training (**Figure 4.7M**; $p = 0.044$, all other p's > 0.065). In contrast, no interactions were detected in adult-onset mice, with all mice exhibiting a progressive reduction in working memory correct errors with training (**Figure 4.7N**) [Day effect: $F(4.24,186.56) = 4.89$, $p < 0.001$, $\eta 2$ p = 0.100].

Analyses of the data from mice tested in later withdrawal failed to indicate a significant 4-way interaction [**Figures 4.7O– R**; Day × Sex × Age × Drinking History ANOVA: $p = 0.168$, $\eta 2p = 0.064$]. However, a significant Day x Drinking History interaction was detected [$F(4.429,389.719) = 6.02$, $p < 0.001$ $\eta 2p = 0.064$] that reflected *fewer* working memory correct errors committed by binge- versus water-drinking mice on the first two days of radial arm maze training (**Figure 4.7S**; $p < 0.001$)–a result suggestive of better working memory performance in binge- versus water-drinking mice. However, it is notable that the time-course of working memory errors committed by binge-drinking mice during later withdrawal was relatively flat (**Figure 4.7S**); in fact, binge- drinking mice committed significantly *more* working memory correct errors later during training than at the start of training (**Figure 4.7S**; day 2 vs. days 3–5, all p's < 0.027). In contrast, the number of working memory correct errors committed by water- drinking mice declined progressively over the course of training, indicative of intact learning (**Figure 4.7S**; day 2 vs. subsequent days, all $p's < 0.046$).

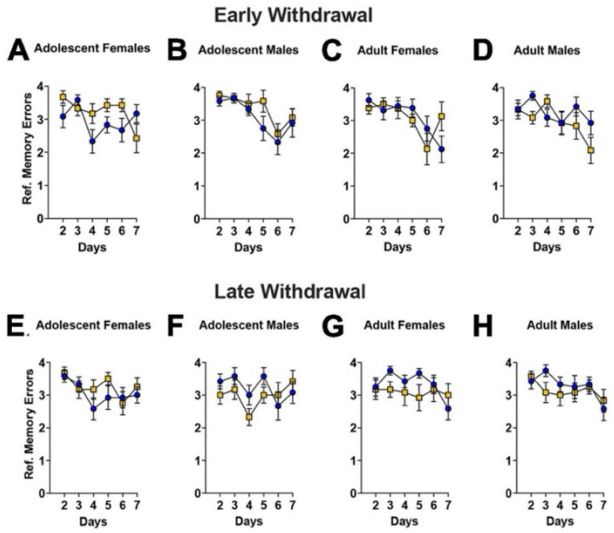
4.3.6.3. Working Memory Incorrect Errors

No significant Day × Sex × Age × Drinking History interaction was detected for the number of working memory incorrect errors committed by the mice tested in early withdrawal (**Figures 4.7T–W**; 4-way ANOVA: $p = 0.588$, $\eta 2p = 0.008$). However, a significant Day × Age x Drinking History interaction was found for this time-point [$F(4.26,374.68) = 2.76$, $p = 0.018$, $\eta 2p = 0.030$]. Deconstruction of this interaction along the Age factor indicated a significant Day x Drinking History interaction for both age groups [ANOVA for adolescent-onset: $F(4.31,198.12) = 2.84$, $p = 0.017$, $\eta 2p = 0.058$; ANOVA for

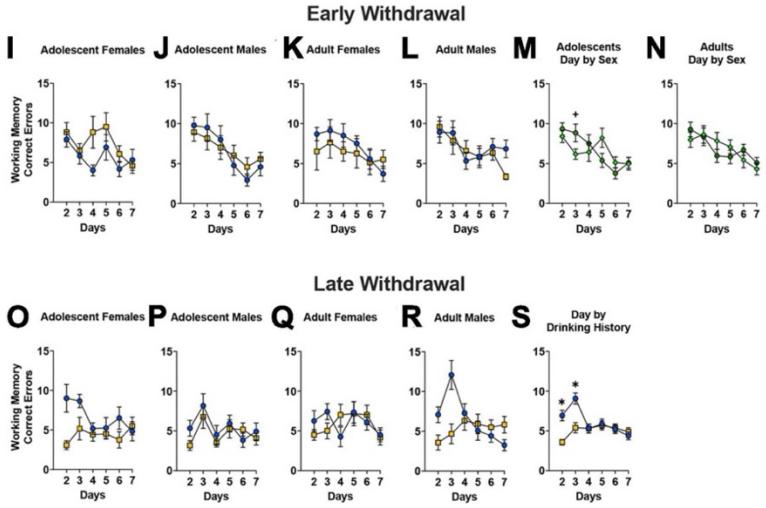
adult-onset: $F(4.03,185.41) = 2.91$, $p = 0.014$, $\eta 2p = 0.060$]. On days 2, 4, and 5, adolescent-onset binge-drinking mice made more working memory incorrect errors versus their water controls (**Figure 4.7X**; Day 2: $p = 0.005$; Day 4: $p = 0.023$; Day 5: $p = 0.034$). In contrast, adult-onset binge- drinking mice committed fewer working memory incorrect errors than water controls but only on day 3 (**Figure 4.7Y**; $p = 0.014$). As depicted in **Figures 4.7X, Y**, the number of working memory incorrect errors declined progressively in both water- and binge- drinking mice, indicative of learning in all groups when tested at the earlier time-point.

For the mice tested in later withdrawal, no significant Day × Age × Sex × Drinking History interaction was found for the number of working memory incorrect errors [**Figures 4.7Z–C**'; 4-way ANOVA $p = 0.267$, $\eta 2p = 0.014$]. However, a significant Day x Drinking History interaction [$F(4.16,365.85) = 2.68$, $p = 0.030$, $\eta 2p = 0.029$] was detected that reflected a lower number of working memory incorrect errors in binge- versus water-drinking mice, but only on day 2 of training (**Figure 4.7D**'; $p < 0.001$, all other p's > 0.092). Consistent with the data for the number of working memory correct errors, water-drinking controls tested in later withdrawal exhibited a progressive decline in the number of working memory incorrect errors with training (**Figure 4.7D**'; days 2 and 3 vs. 4–7, all p's < 0.041), while the time-course of behavior was flat in binge-drinking animals (**Figure 4.7D**'; day 2 < day3, $p = 0.032$), indicative of little to no learning.

Reference Memory Errors



Working Memory Correct Errors



Working Memory Incorrect Errors

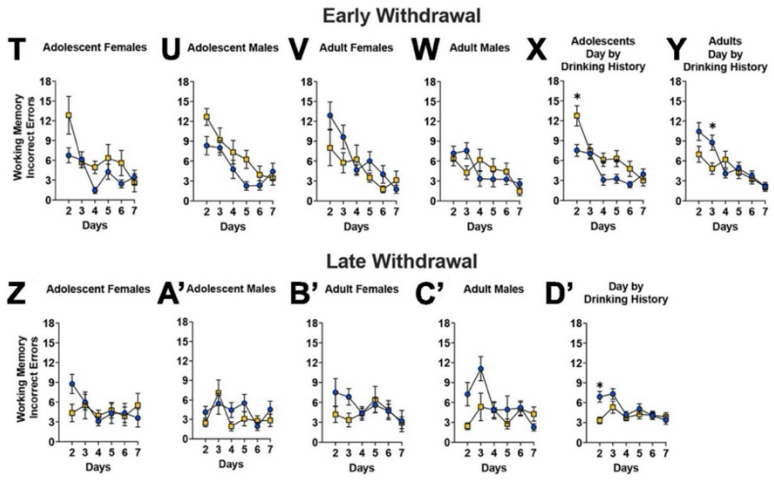


Figure 4.7. Depiction of the results of the Day × Sex × Age × Drinking History mixed-model ANOVAs evaluating reference memory, working memory correct and incorrect errors in the Radial Arm Maze. (A–D) For mice tested in early withdrawal (top), a significant Day by Sex by Age by Drinking History interaction was detected for the number of reference memory errors during the first week of radial arm maze training testing. **(A,C)** In female mice, a significant Day by Age by Drinking History interaction was found and follow up analyses indicated a significant Day by Drinking History interaction for adolescent females. However, no statistically significant drinking history differences were noted for the adolescent females on any of the training days [females: adolescent H20 (n = 12); adolescent EtOH (n = 12); adult H2O (n = 16); adult EtOH (n = 8)]. **(B,D)** For male mice, a Day by Age interaction was observed, reflecting more errors by adult versus adolescent males on day 6 of training only irrespective of drinking history [males: adolescent H2O (n = 12); adolescent EtOH (n = 12); adult H2O (n = 12); adult EtOH (n = 12)]. **(E–H)** For the mice tested in later withdrawal, a significant Day by Age interaction was found on day 4 of training, with more errors by adults than adolescents. For WD30, sample sizes were the following: **(E)** H2O (n = 12), EtOH (n = 12); **(F)** H2O (n = 12), EtOH (n = 12); **(G)** H2O (n = 12), EtOH (n = 12); **(H)** H2O (n = 12), EtOH (n = 12). Note that interactions that do not include Drinking History as a factor have not been included in panels **(A–H)**. **(I–L)** For mice tested in early withdrawal, there was a significant Day by Sex by Age by Drinking History interaction for working memory correct errors committed in the radial arm maze. The samples sizes are the same as panels **(A–D)**. When collapsed along the Age factor, **(M)** a significant Day by Sex interaction for adolescent mice indicated that males committed more errors on day 3 of training [females (n = 24), males (n = 24)]. **(N)** However, only a main effect of Day was observe for adult mice [females (n = 24), males (n = 24)]. **(O–S)** For WD30 mice, no significant 4-way interaction was found. The samples sizes are the same as panels **(E–H)**. **(S)** There was a significant Day by Drinking History interaction during late withdrawal that indicated binge-drinking mice committed fewer errors on the first two days [H2O (n = 48), EtOH (n = 48)]. **(T–W)** No significant Day by Sex by Age by Drinking History interaction was detected for the number of working memory incorrect errors committed by the mice tested in early withdrawal. The sample sizes are the same as panels **(A–D)**. However, deconstruction of the significant Day by Age by Drinking History interaction along the Age factor indicated that **(X)** adolescent-onset binge-drinking mice made more errors on certain days [H2O (n = 24), EtOH (n = 24)], while **(Y)** adult-onset binge-drinking mice committed fewer errors only on day 3 [H2O (n = 28), EtOH (n = 20)]. **(Z–C')** For the mice tested in later withdrawal, no significant Day by Age by Sex by Drinking History interaction was found. The sample sizes are the same as panels **(E–H)**. **(D')** However, a significant Day by Drinking History interaction was detected that reflected a progressive decline in working memory incorrect errors in water-drinking animals versus the relatively flat time-course of errors exhibited by binge-drinking mice [H2O (n = 48), EtOH (n = 48)]. The data represent the means ± SEMs of the number of mice indicated above. *$p < 0.05$, EtOH vs. H2O; $^{+}p < 0.05$, Female vs. Male.

4.3.6.4. Chaining Behavior

The Day × Sex × Age × Drinking History ANOVA for the mice tested in early withdrawal indicated in no significant four-way interaction for chaining behavior [**Figures 4.8A–D**; 4- way ANOVA: $p = 0.184$, $\eta 2p = 0.017$]. However, a significant Day × Sex × Drinking History interaction was observed [$F(4.26,374.80) = 3.02$, $p = 0.016$, $\eta 2p = 0.033$]. Deconstruction of this interaction along the Sex factor yielded a significant Day × Drinking History interaction for the female mice (**Figure 4.8E**) [$F(3.69,169.50) = 3.96$, $p = 0.005$, $\eta 2p = 0.079$]. As illustrated (**Figure 4.8E**), binge-drinking females exhibited more chaining behavior than their water controls on day 4 ($p = 0.002$) and day 5 ($p = 0.008$) of training. No significant interactions were detected for the male mice tested in early withdrawal (**Figure 4.8F**) [ANOVA: $p = 0.416$, $\eta 2p = 0.021$]. As illustrated (**Figure 4.8F**), all males exhibited a training-dependent reduction in the amount of chaining behavior [Day effect: $F(4.01,184.40) = 21.63$, $p < 0.001$, $\eta 2p = 0.320$; *post-hoc* tests, days 2 and 3 versus days 4–7, all p's < 0.010].

The analyses of the data for the mice tested in later withdrawal failed to detect a significant Day × Age × Sex × Drinking History interaction [**Figures 4.8G–J**; 4-way ANOVA, $p = 0.338$, $\eta 2p = 0.010$]. However, a significant Day x Sex interaction was observed [$F(4.16,365.77) = 2.57$, $p = 0.036$, $\eta 2p = 0.028$] that reflected more chaining episodes in females versus males on day 2 of training, while males exhibited more chaining episodes on day 4 [**Figure 4.8K**; Day 2: $p = 0.053$; Day 4: $p = 0.026$]. As illustrated in **Figure 4.8K**, male mice exhibited a progressive decline in the amount of chaining across the first week of testing, indicative of a shift from non-spatial to spatial learning strategies [Day 2 vs., Days 4 –6: p's < 0.033]. While chaining behavior declined early during training in the

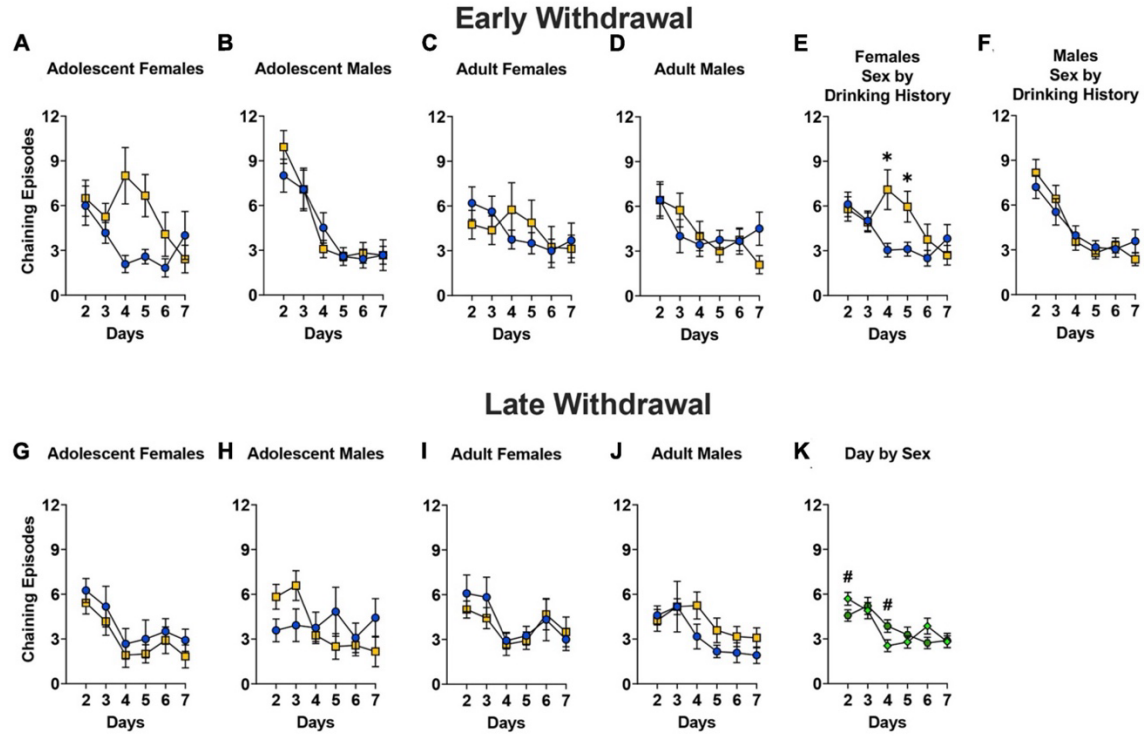
females tested in later withdrawal (**Figure 4.8K**; days 2 and 3 vs. days 5–7; all *p's* < 0.003), this behavior plateaued, with females exhibiting more chaining on day 6, relative to day 4 (*p* = 0.032) and day 7 (**Figure 4.8K**; *p* = 0.037).

4.3.6.5. Time to Complete the Maze

No significant interactions between Day × Sex × Age × Drinking History were detected for the total time taken to find all the platforms in the radial arm maze when the mice were tested in early withdrawal [**Figures 4.8L–O**); all ANOVA *p's* > 0.147]. All mice exhibited a progressive decline in the amount of time taken to complete the maze [Day effect: $F(4.15, 364.98) = 42.03$, $p < 0.001$, $\eta 2p = 0.323$; *post-hoc* tests for all groups, all *p's* < 0.030].

No significant 4-way interaction was observed with respect to the time taken by mice to complete the radial arm maze during later withdrawal [**Figures 4.8P–S**; Day × Sex × Age × Drinking History ANOVA: $p = 0.206$, $\eta 2p = 0.018$). However, a significant Day x Group interaction [$F(4.08, 358.22) = 4.96$, $p = 0.001$, $\eta 2p = 0.053$] was found that reflected a shorter time taken by binge- versus water-drinking mice on days 2 and 3 of training [**Figure 4.8T**; Day 2 $p < 0.001$; Day 3 $p = 0.052$). As illustrated in **Figure 4.8T**, the WD30 water-drinking mice exhibited a progressive decline in the time taken to complete the maze, consistent with learning (day 2 vs. days 5–7; all *p's* < 0.002). In contrast, the time-course for this variable exhibited an inverted U-shape in the binge-drinking mice tested in later withdrawal, with the longest latency to complete the maze observed on day 4 of training (**Figure 4.8T**; all *p's* < 0.043).

Chaining Behavior



Time to Complete Maze (sec)

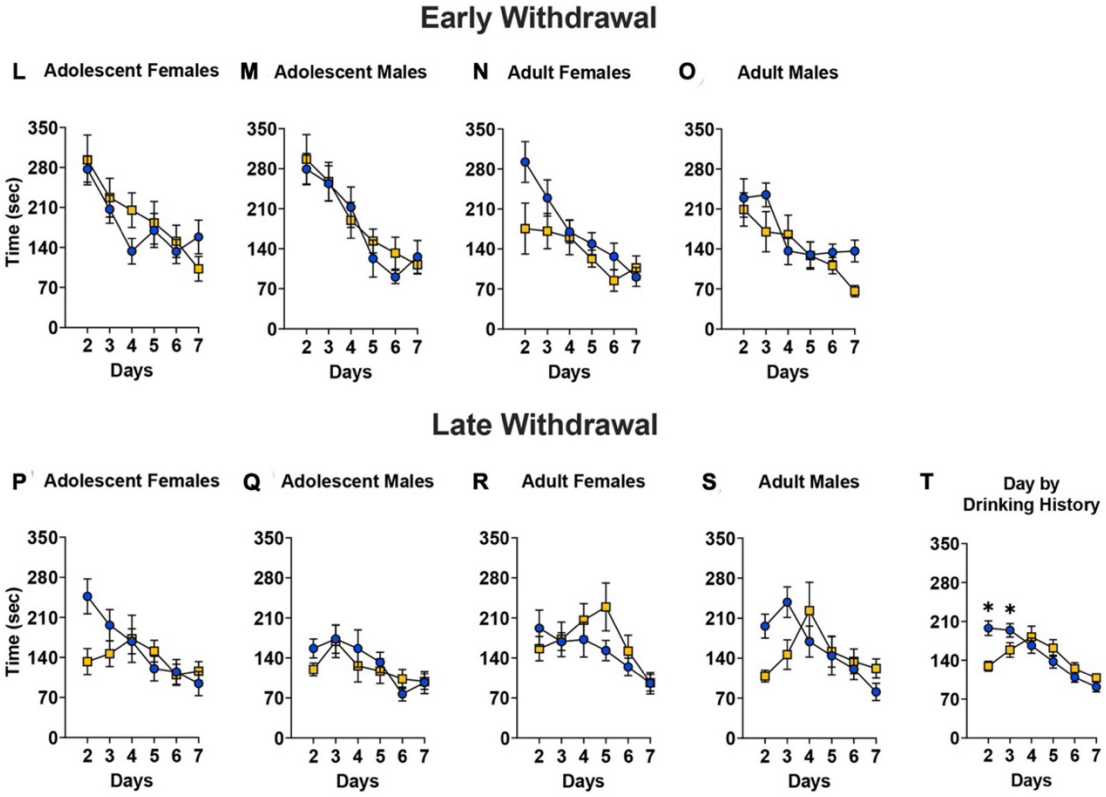


Figure 4.8. Depiction of the results of the Day × Sex × Age × Drinking History mixed-model ANOVAs evaluating non-spatial navigation (chaining episodes) and time taken to navigate the Radial Arm Maze. (A–D) No significant 4-way interaction in mice tested in early withdrawal. For early withdrawal, the sample sizes were as follows: **(A)** H2O (*n* = 12), EtOH (*n* = 12); **(B)** H2O (*n* = 12), EtOH (*n* = 12); **(C)** H2O (*n* = 16), EtOH (*n* = 8); **(D)** H2O (*n* = 12), EtOH (*n* = 12). **(E)** However, a significant Day by Sex by Drinking History interaction was detected that reflected more chaining behavior by binge-drinking females than water controls on days 4 and 5 [H2O (*n* = 28), EtOH (n = 20)]. **(F)** No significant interaction was detected for males tested in early withdrawal [H2O (*n* = 24), EtOH (*n* = 24)]. **(G–J)** In mice tested in later withdrawal, no significant 4-way interaction was found. For late withdrawal, the sample sizes were as follows: **(G)** H2O (*n* = 12), EtOH (*n* = 12); **(H)** H2O (*n* = 12), EtOH (*n* = 12); **(I)** H2O (*n* = 12), EtOH (*n* = 12); **(J)** H2O (*n* = 12), EtOH (*n* = 12). **(K)** However, a significant Day by Sex interaction was detected that reflected sex differences in chaining on day 2 and 4 of training [females (*n* = 48), males (*n* = 48)]. The data represent the means ± SEMs for the number of mice indicated above. **(L–O)** For the mice tested in early withdrawal, there were no significant Day by Sex by Age by Drinking History interactions on the total time to complete the maze, and all mice showed improvement in maze completion over time. The sample sizes are the same as for panels **(A–D)**. **(P–S)** For the mice tested in later withdrawal, no significant 4-way interaction was detected. The sample sizes are the same as for panels **(G–J)**. **(T)** However, a significant Day by Group interaction was noted, which reflected a shorter latency to complete the maze on days 2 and 3 by binge-drinking mice [H2O (*n* = 48), EtOH (*n* = 48)]. The data represent the means ± SEMs for the number of mice indicated above. **p* < 0.05, EtOH vs. H2O; $^{\#}p$ < 0.05, adolescents vs. adults.

4.3.7. Replicate Testing for Alcohol Withdrawal-Induced Negative Affect

An analysis of the average total alcohol consumed over the 2- week drinking period indicated a significant Sex × Age interaction [$F(1,23) = 6.33$, $p = 0.021$; η2p = 0.240]. In this replicate study, the interaction reflected higher alcohol intake by male adolescent mice versus their adult controls [$t(10) = 6.28$, $p < 0.001$], with no age difference detected for the relatively high alcohol intake exhibited by female subjects (**Figure 4.9A**; *t*-test, $p = 0.858$).

Light-Dark Shuttle Box

4.3.7.1. Latency to Enter the Light-Side

Under these more insulated testing conditions, we detected two significant interactions with respect to the latency to first enter the light-side of the light-dark shuttle box (**Figure 4.9B**). As illustrated in **Figure 4.9C**, a Sex × Age interaction [$F(1,47) = 5.57$, $p = 0.023$; $\eta 2p = 0.122$] reflected a shorter latency of adolescent versus adult males to enter the light-side [$t(22) = 2.24$, $p = 0.035$], with no age difference observed in females (*t*-test, $p = 0.516$). We also detected a significant Age X Drinking interaction for this variable (**Figure 4.9D**) [$F(1,47) = 5.59$, $p = 0.023$; $\eta 2p = 0.123$]. Although inspection of **Figure 4.9D** suggested that this interaction reflected specifically an alcohol-induced increase in the latency of adult mice to first enter the light-side, water-alcohol differences were not detected for either age group (*t*-tests, *p*'s > 0.158). Rather, the Age × Drinking interaction reflected a longer latency to enter the light side by alcohol-experienced adults versus their adolescent counterparts [$t(22) = 2.28$, $p = 0.032$], with no age difference noted for water controls (**Figure 4.9D**; *t*-test, $p = 0.697$).

4.3.7.2. Light Side Time and Entries

A summary of the data for the time spent on the light side is depicted in **Figure 4.9E**. A significant Age × Drinking interaction was also detected for this variable [$F(1,47) = 3.63$, $p = 0.064$; $\eta 2p = 0.083$], which reflected less time spent by alcohol-experienced adults versus their water controls [$t(22) = 3.14$, $p = 0.055$] with no water-alcohol differences detected in adolescent mice (**Figure 4.9F**; *t*-test, $p = 0.499$). Although it appeared that adult female alcohol- experienced mice entered the light-side fewer times than their water controls (**Figure 4.9G**), we detected only an overall effect of age with respect to this variable, with

adults spending less time in the light-side than adolescents (**Figure 4.9H**) [Age effect: $F(1,47) = 2.93$, $p = 0.095$; η2p = 0.068; other p's > 0.133; η2p's < 0.056].

Marble-Burying

4.3.7.3. Latency to Bury

A summary of the data for the latency to begin marble burying is provided in **Figure 4.9I**. Under the more insulated testing conditions, we detected a significant Age × Sex interaction for the latency to begin marble burying [$F(1,47) = 3.68$, $p = 0.062$; η2p = 0.084] that reflected a longer latency of adolescent versus adult males [$t(22) = 2.71$, $p = 0.013$], with no age difference noted for females (**Figure 4.9J**; t-test, $p = 0.885$). We also detected an overall Drinking effect [$F(1,47) = 3.46$, $p = 0.070$; η2p = 0.080] that reflected a shorter latency to bury in alcohol-experienced mice versus water controls, irrespective of the animals' age or sex (**Figure 4.9K**; Drinking interactions, all p's > 0.149; η2p's < 0.052).

4.3.7.4. Marbles Buried

In contrast, we detected only an overall Age effect with respect to the number of marbles buried (**Figure 9L**) [$F(1,47) = 5.482$, $p = 0.024$; η2p = 0.121; other p's > 0.117; η2p's < 0.061], that reflected more marbles buried by adult versus adolescent mice (**Figure 4.9M**).

Forced Swim Test

4.3.7.5. Latency to First Immobile Episode

As depicted in **Figure 4.9N**, there was considerable variability in the latency to first float in the forced swim test even when extreme outliers were removed. However, we did detect a significant Sex × Drinking History interaction (**Figure 4.9O**) [$F(1,45) = 70.15$, $p = 0.050$; $\eta 2p = 0.825$]. This interaction reflected a longer latency to float by alcohol-experienced males versus their water controls [$t(22) = 2.022$, $p = 0.056$], with no significant alcohol- water difference detected in females (*t*-test, $p = 0.235$). No Age effect or interactions were detected for this variable (p's > 0.208; $\eta 2p < 0.650$).

4.3.7.6. Number and Duration of Immobility

In contrast to the latency to float, we detected no alcohol or sex effects for the time spent floating (**Figure 4.9P**; p's > 0.187; $\eta 2p < 0.044$) or the number of floating episodes (**Figure 4.9R**; p's > 0.282; $\eta 2p < 0.029$) or in the forced swim test. Instead, we detected only main Age effects for both variables (**Figures 4.9O, S**) [for float episodes, Age effect: $F(1,47) = 8.73$, $p = 0.005$; $\eta 2p = 0.179$; for float time (sec), Age effect: $F(1,47) = 6.86$, $p = 0.012$; $\eta 2p = 0.146$] that reflected less floating-related behavior in adult vs. adolescent mice.

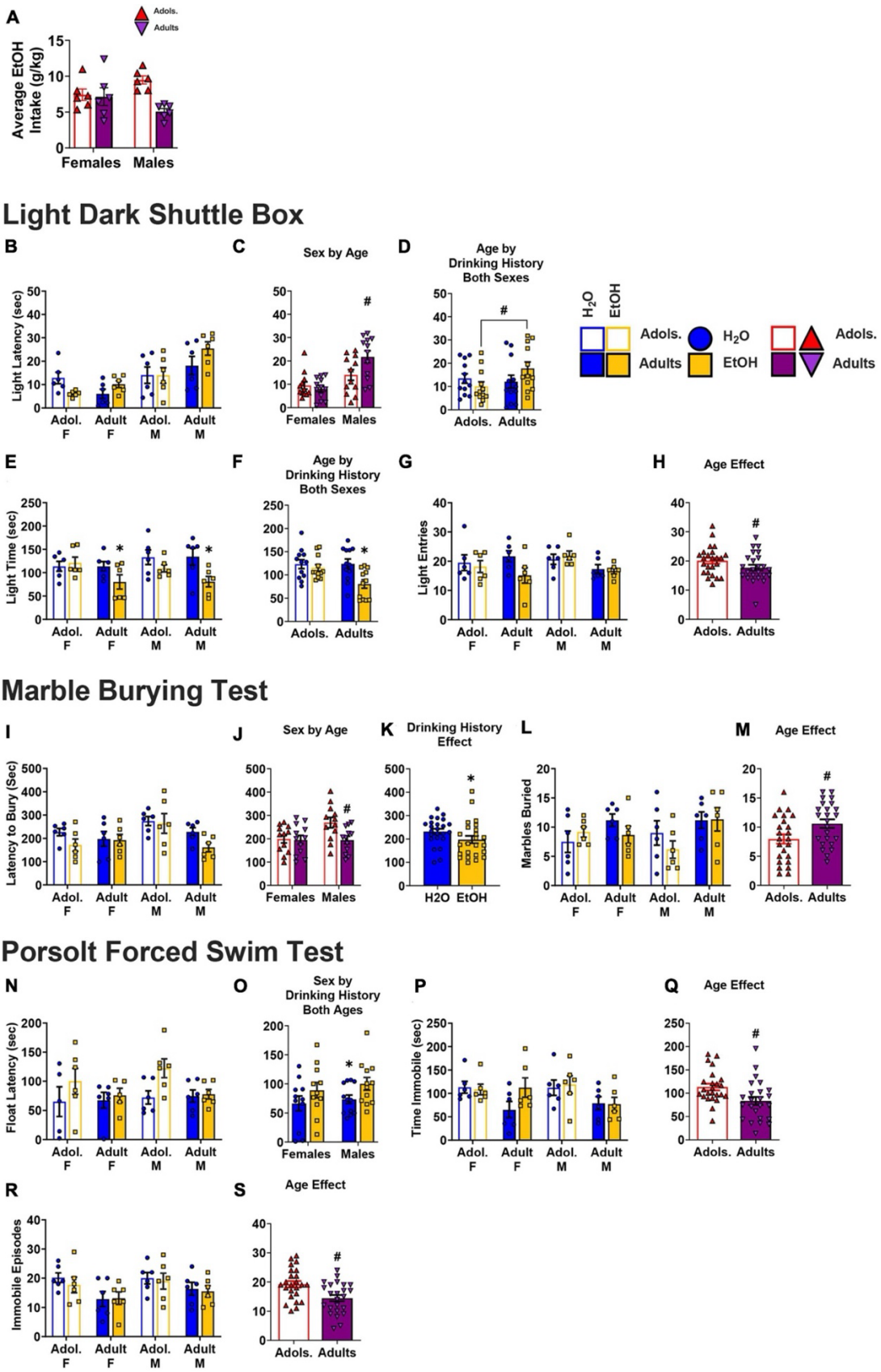

A
Adols.
Adults
Average EtOH Intake (g/kg)
Females
Males
Light Dark Shuttle Box
B
Light Latency (sec)
Adol. F
Adult F
Adol. M
Adult M
C
Sex by Age
Females
Males
D
Age by Drinking History Both Sexes
Adols.
Adults
H2O
EtOH
E
Light Time (sec)
F
Age by Drinking History Both Sexes
G
Light Entries
H
Age Effect
Marble Burying Test
I
Latency to Bury (Sec)
J
Sex by Age
K
Drinking History Effect
H2O
EtOH
L
Marbles Buried
M
Age Effect
Porsolt Forced Swim Test
N
Float Latency (sec)
O
Sex by Drinking History Both Ages
P
Time Immobile (sec)
Q
Age Effect
R
Immobile Episodes
S
Age Effect

Figure 4.9. Depiction of the results from Study 2. (A) In the replicate study, a significant age x sex interaction was detected for the amount of alcohol consumed (n = 6/sex/age), that reflected more alcohol intake by adolescent versus adult males. **(B)** Summary of the data for the latency to first enter the light-side of the light-dark shuttle box on WD 1 (n = 6/sex/age/drinking history). The ANOVA conducted on this variable revealed a Sex by Age interaction [**(C)**; n = 12/sex/drinking history] and an Age by Drinking History interaction [**(D)**; n = 12/age/drinking history]. **(E)** Summary of the data for the time spent in the light side (in sec), highlighting a main drinking history effect in adult mice (n = 6/sex/age/drinking history). **(F)** The ANOVA conducted on this variable indicated also an Age × Drinking History interaction that reflected H2O-EtOH differences in adult mice (n = 12/age/drinking history). **(G)** Summary of the number of entries into the light side of the shuttle box (n = 6/sex/age/drinking history). **(H)** The ANOVA indicated fewer light-side entries by adults vs. adolescents (n = 24/age). **(I)** Summary of the data for the latency to begin marble-burying (n = 6/sex/age/drinking history). The ANOVA indicated a Sex × Age interaction [**(J)**; n = 12/sex/age], as well as a main Drinking History effect [**(K)**; n = 24/drinking history]. **(L)** Summary of the data for the number of marbles buried (n = 6/sex/age/drinking history). **(M)** The ANOVA indicated that adults buried more marbles than adolescent mice (n = 24/age). **(N)** Summary of the data for the latency to first floatin in the forced swim test (n = 6/sex/age/drinking history) [females: adolescent-H20 (n = 5); adolescent-EtOH (n = 6); adult-H2O (n = 6); adult-EtOH (n = 5); males: n = 6/age/drinking history]. **(O)** The ANOVA revealed a Sex × Drinking History inaction, but no specific H2O-EtOH differences were detected [female-H2O (n = 11); female-EtOH (n = 11); male-H2O (n = 12); male-EtOH (n = 12]. **(P)** Summary of the data for the time spent immobile (n = 6/sex/age/drinking history). **(Q)** The ANOVA indicated less time immobile in adult versus adolescent mice (n = 24/age). **(R)** Summary of the data for the number of immobile episodes (n = 6/sex/age/drinking history), the ANOVA for which indicated fewer immobile episodes in adult versus adolescent mice [**(S)**; n = 24/age]. The data are presented as the means ± SEMs for the respective number of mice indicated above. $^{*}p < 0.05$ H2O vs. EtOH; $^{\#}p < 0.05$, adolescents vs. adults (main Age effect).

4.4. Discussion

The present study was designed to expand upon a recent report from our group describing weak interactions between a sub-chronic (i.e., 2 week) history of binge-drinking, the age of drinking-onset and sex in the affective consequences of alcohol assayed at 1 versus 70 days withdrawal (Jimenez Chavez et al., 2020). The results of this prior study (Jimenez Chavez et al., 2020) contrasted with earlier reports of robust, age-dependent, effects in the marble-burying, light-dark box and forced swim tests (Lee et al., 2016, 2017a,b, 2018a,b; Szumlinski et al., 2019). As these latter studies employed a single sex and tested for negative

affect at 1 versus 30 days withdrawal, herein, we segregated the testing of our male and female mice on WD1 and WD30 to reduce the influence of chemosensory social stimuli from the opposite sex on behavior. Based on a recent study of older mice (>6 months of age) indicating sex differences in alcohol-induced cognitive impairment (Jimenez Chavez et al., 2022), as well as published work from other groups indicating that a history of alcohol-drinking during adolescence can accelerate the onset of cognitive decline (e.g., Ledesma et al., 2021; Van Hees et al., 2022), we also tested for interactions between our subject factors with respect to spatial learning and memory in the Morris water maze, as well as reference and working memory in the radial arm maze. Although we detected some affective and cognitive effects of binge-drinking, the group differences were not as robust as in prior work when a single sex was tested. Thus, we also conducted an additional study to best mimic the procedural conditions of our prior work (i.e., Lee et al., 2016, 2017b, 2018a), in which we single-housed water controls during drinking procedures and behavioral testing was conducted in series in distinct procedural rooms.

4.4.1. Robust Binge-Drinking for 2 weeks Elicits Relatively Few Effects on Negative Affect During Alcohol Withdrawal

Dependent variable	Study 1: WD 1 and 30		Study 2: WD1 only
Binge drinking			
Average Total Intake	**adolescent** > **adults**females > males		**adolescents** > **adults** (males only)
BACs	adolescent > adultsfemales > males		ND
	Early Withdrawal	**Late Withdrawal**	**Early Withdrawal**
Tests for negative affect			
Latency to enter the light side	EtOH = H2O	EtOH = H2O	EtOH = H2O
Time spent in the light side	EtOH > H2O (adolescents only) **EtOH < H2O (adults only)**	EtOH = H2O	**EtOH < H2O (adults only)**
Entries into the light side	EtOH = H2O	EtOH = H2O	EtOH = H2O
Latency to bury marbles	ND	ND	EtOH < H2O
Number of marbles buried	EtOH = H2O	EtOH = H2O	EtOH = H2O
Latency to immobility	EtOH > H2O	EtOH = H2O	EtOH = H2O
Time spent immobile	EtOH > H2O (adolescent males only) EtOH < H2O (adult males only)	EtOH = H2O	EtOH = H2O
Immobile Episodes	EtOH > H2O (adult females only) EtOH > H2O (adolescent males only)	EtOH < H2O (adolescents only) EtOH < H2O (males only)	EtOH = H2O
Morris water maze			
Latency to platform during the flag test	EtOH = H2O	EtOH = H2O	
Latency to platform during acquisition	EtOH = H2O	EtOH = H2O	
Latency to platform during probe test	EtOH < H2O (adolescents only)	EtOH = H2O	
Entries to platform location during probe test	EtOH = H2O	EtOH < H2O (adolescents only) EtOH > H2O (adults only)	
Time spent in the NE quadrant	EtOH = H2O	EtOH = H2O	
Latency to new platform location	EtOH = H2O	EtOH = H2O	
Radial arm water maze			
Reference memory errors	EtOH = H2O	EtOH = H2O	
Working memory correct errors	EtOH = H2O	Days 2 and 3: EtOH < H2O	
Working memory incorrect errors	Days 2, 4 and 5: EtOH > H2O (adolescents only) Day 3: EtOH < H2O (adults only)	Day 2: EtOH < H2O	
Chaining episodes	Days 4 and 5: EtOH > H2O (females only)	EtOH = H2O	
Time to locate all platforms	EtOH = H2O	Days 2 and 3: EtOH < H2O	

EtOH-Water differences in behavior that were consistent across the two studies and/or that align with prior published studies by our group are bolded. ND indicates not determined. The mice in Study 2 were only assayed for negative affect.

Table 4.5. Summary of the effects of a 2-week history of binge-drinking upon our measures of negative affect and cognition.

A summary of the effects of alcohol withdrawal on our behavioral measures from our two studies is presented in **Table 4.5**. As expected (Finn et al., 2010; Strong et al., 2010; Wilsnack et al., 2018; Szumlinski et al., 2019; Jimenez Chavez et al., 2020, 2022), the female mice in the larger study binge-drank more alcohol than males and exhibited higher BACs (**Figure 4.2B**). Also as expected (Moore et al., 2010; Melón et al., 2013; Lee et al., 2016, 2017b, 2018a; Szumlinski et al., 2019; Jimenez Chavez et al., 2020), adolescents consumed more alcohol and attained higher BACs than their adult counterparts (**Figure 4.2A**). Moreover, BACs on the day of sampling were at or above the NIAAA 80 mg/dL criterion for binge- drinking (National Institute on Alcohol Abuse and Alcoholism [NIAAA], 2004) and BACs correlated with alcohol intake, with adult males exhibiting the lowest intakes/BACs, and adolescent females exhibiting the highest intakes/BACs (**Figure 4.2C**). However, in the smaller scale study (n = 6/sex/age/group), the sex and age differences were less robust, owing to the relatively high alcohol intake of the adolescent males (**Figure 4.9A**).

However, as observed in our prior large study of sex by age interactions in alcohol withdrawal-induced negative affect (Jimenez Chavez et al., 2020), we detected very few alcohol or -age-related differences in negative affect in either of the studies presented herein (see summary in **Table 4.5.**). Thus, we twice failed to replicate the robust alcohol by age by withdrawal interactions detected for the majority of our dependent variables in our earlier studies employing a single sex (Lee et al., 2016, 2017b, 2018a,b; Szumlinski et al., 2019). As chemosensory social stimuli from females can affect anxiety-like behavior in males (Aikey et al., 2002; Fernández- Guasti and Martínez-Mota, 2005; Frye et al., 2008), both of the

experiments herein tested males and females on different days to mitigate this influence. Thus, gonadal pheromones from mice of the opposite sex during testing cannot readily account for the relatively weak effects of alcohol withdrawal upon our measures of negative affect in the present studies. Likewise, as female mice have historically been housed in the same colony room as male mice, either under ventilated or filter-top-type caging over the years that we have been conducting binge-drinking studies in mice, it is also unlikely that gonadal pheromones from mice of the opposite sex in the colony room can account for the relatively weak effects of alcohol observed in the present studies.

It is interesting to note that we detected more male-selective effects of alcohol withdrawal in the present large-scale study (**Table 4.5**), compared to that previous employing concurrent testing of male and female subjects (Jimenez Chavez et al., 2020). As highlighted in **Table 4.5**, male-selective alcohol-water differences were noted for the entries into the light-side in the light-dark shuttle box test (WD1), the time spent immobile in the forced swim (WD1), and the number of immobile episodes (both WD1 and WD30), while female-selective alcohol-water differences were noted for the number of immobile episodes (WD1) and the number of marbles buried (WD30) (**Table 4.5**). Further, the fact that some sex by age interactions for our measures of negative affect were observed when male and female mice are segregated during testing for negative affect indicates that a segregation strategy may prove more fruitful for detecting such interactions be more optimal for detecting sex-selective effects than concurrent testing of both sexes. Admittedly, the smaller scale replicate study was likely insufficiently powered to detect sex by alcohol interactions as we detected only trends for sex-selective alcohol effects (**Figure 4.9**). This being said, Sex by Age

interactions were noted for the latency to enter the light- side of the light-dark shuttle box (**Figure 4.9C**) and the latency to being burying marbles (**Figure 4.9J**), in which males exhibited the age-related difference in behavior. However, the simple fact remains that three of our sex differences studies to date (Jimenez Chavez et al., 2020; present study) have yielded less robust and consistent alcohol effects on anxiety- and depressive-like behaviors than our earlier single-sex studies. While it might be argued that the group- housing procedure employed for water control mice in the present larger scale study and that previous (Jimenez Chavez et al., 2020) may have confounded their results, age by alcohol interactions were apparent in earlier single-sex studies using comparable group- housed water-drinking procedures (Lee et al., 2018a; Szumlinski et al., 2019). Moreover, individually housing both the water- and alcohol-drinking mice in the follow-up study herein did not improve experimental outcomes (see **Table 4.5**), despite the study being sufficiently powered to detect alcohol by age interactions (n = 12/age/drinking history).

At the time we completed the larger scale study herein, we considered two additional procedural factors that might account for the discrepancy across our sex difference (Jimenez Chavez et al., 2020; present study) versus single-sex studies to date (e.g., Lee et al., 2016, 2017a,b, 2018a,b; Szumlinski et al., 2019): (1) the research personnel conducting the study and (2) the location of the behavioral laboratory. However, as both studies of one or both sexes are labor-intensive, they have always been conducted by teams of researchers such that the mice are handled by multiple, different, researchers throughout drinking and are only tested for negative affect by individuals familiar to the mice, with the goal of minimizing experimenter-induced anxiety-like behavior. We followed a similar “team” approach in the

larger scale study herein, while both the drinking and behavioral testing procedures employed in the smaller scale study was conducted by a single researcher. Thus, it would not appear that our "team approach" is a major driver of our failure to detect age by alcohol interactions when both sexes are studied.

A more plausible explanation relates to the locations of the colony rooms in which mice consumed alcohol/water and the procedural space employed for behavioral testing. The mice in all our earlier studies (Lee et al., 2016, 2017a,b, 2018a,b; Szumlinski et al., 2019) were housed and drank alcohol in a small satellite vivarium, with testing conducted in several, small, distinct procedural rooms dedicated to a specific behavioral test that were located outside of the vivarium. While the same behavioral equipment and procedures for assaying negative affect continue to be employed, the three most recent studies from our group examining for age by sex interactions in alcohol withdrawal-induced anxiety (Jimenez Chavez et al., 2020, 2022; present study) were all conducted in the main campus vivarium, in large procedural rooms housing multiple apparati, during which groups of mice undergo different tests concurrently in the same room (i.e., tests for marble-burying conducted on the bench along the right side of the room, with tests for light- dark box conducted on the bench along the left side of the room). To minimize the noise associated with daily vivarium routines, we only tested mice for negative affect on weekends when vivarium staff was minimal and the general vivarium traffic low. However, the modular nature of our current behavioral testing space may not be ideal for testing anxiety- and depressive-like behavior in mice. To probe this possibility, each behavioral assay in the smaller, follow-up, study was conducted in distinct rooms within the main campus vivarium and the mice underwent the

behavioral procedures in series. As illustrated in **Figure 9**, the procedural modifications in the second study were sufficient to unmask age differences and/or age by sex interactions for our light-dark shuttle-box and forced swim measures that were not apparent in the larger scale study (see **Figures 4.3**, **4.5**, respectively). However, as highlighted in **Table 4.5**, we detected fewer alcohol- related effects in the follow-up study than the larger original study. Unfortunately, as our small satellite vivarium no longer exists, we cannot directly compare outcomes from experiments conducted in the main versus satellite vivaria. Given this, we can conclude that segregating the sexes during behavioral testing and sample size, but not necessarily the involvement of a single versus a team of experimenters, the employ of single versus group-housing of water controls and serial versus concurrent behavioral testing appear to influence the manifestation of negative affect during alcohol withdrawal.

4.4.2. Robust Binge-Drinking for 2 weeks Elicits a Few Signs of Mild Cognitive Impairment During Alcohol Withdrawal

The extant human (e.g., Squeglia et al., 2009, 2011a,b; Novier et al., 2015; Cservenka and Brumback, 2017; Huang et al., 2018; Ledesma et al., 2021) and rodent (Salling et al., 2016; Grifasi et al., 2019; Hoffman et al., 2019; Jimenez Chavez et al., 2022; Van Hees et al., 2022) literature indicates that a history of excessive drinking can accelerate cognitive decline and associated neuropathology, with adolescent female binge-drinking humans exhibiting greater neurocognitive anomalies than their male counterparts (e.g., Squeglia et al., 2009, 2011a,b). Given the robust sex- and age-related differences in alcohol intake and BACs observed in the present study (**Figure 1**), we predicted that adolescent female mice would exhibit the most robust deficits in cognitive function, potentially exhibiting cognitive

anomalies as young adults. However, as summarized in **Table 4.5**, only one variable across our Morris water maze procedures exhibited alcohol- dependent effects - the number of entries into the former platform location, a measure of spatial recall. These alcohol effects were observed only in later withdrawal (i.e., approximately 60 days following the last drinking day), were of medium effect size (*d*'s∼0.5) and reflected poorer spatial recall by adolescent-onset binge-drinkers, but better spatial recall by adult-onset binge- drinkers (**Figure 4.6N**). No other cognitive measure exhibited an alcohol effect that was selective for adolescent-onset binge-drinkers (**Table 4.5**). Thus, while non-dependence drinking can alter the expression of Alzheimer's Disease-related genes in both adolescent and adult B6 mice (Salling et al., 2016; Hoffman et al., 2019), it may be that a 2-week history of binge-drinking under our 2-h procedures during adolescence is insufficient to accelerate cognitive decline. Alternatively, 3.5 months of age may be too early to detect signs of alcohol-induced cognitive decline in mice with a history of adolescent-onset binge-drinking. Arguing in favor of the former (and against the latter) possibly, Van Hees et al. (2022) recently showed that 10 days of binge-drinking during adolescence under 4-h DID procedures [during which alcohol intakes were approximately double those observed in the present study; see Figure 2C in Van Hees et al. (2022)] is sufficient to induce a deficit in novel object recognition when mice are tested 40 days later. It is also possible that the Morris water maze is less sensitive than other cognitive tasks for the detection of alcohol-induced cognitive decline. Indeed, in our prior study of mature adult and aged mice, we detected very few alcohol-related effects in the Morris water maze, while several measures in the radial arm maze were consistently negatively impacted by an alcohol-drinking history (Jimenez Chavez et al., 2022).

Consistent with this, we detected more alcohol effects in the radial arm maze than in the Morris water maze in the present study (**Table 4.5**). However, in contrast to older mice (Jimenez Chavez et al., 2022), the alcohol-water differences observed in adolescent- and adult-onset binge-drinking mice were not systematic across maze acquisition. For some variables, alcohol effects were observed for 1–2 days during early learning, for other variables they appeared during the middle of the first week of training and no obvious pattern of effect is apparent from the results of specific alcohol- water comparisons as presented in **Table 4.5.** However, a comparison of the shapes of the time-courses for both working memory correct (**Figure 4.6S**) and incorrect errors (**Figure 4.6D**') committed by the binge-drinking mice in later withdrawal argues that a binge- drinking history impairs between-session learning in a manner that is independent of both sex and age of drinking-onset. To the best of our knowledge, this study is the first to examine the effects of a history of binge-drinking during adolescence or younger adulthood on radial arm maze performance. As we know that a month of binge-drinking under our 2-h DID procedures is sufficient to induce sex- and age-selective deficits in radial arm maze performance in older mice (Jimenez Chavez et al., 2022), while binge-drinking large amounts of alcohol (6–8 g/kg/day) over a 10-day period during adolescence is sufficient to induce cognitive deficits in early adulthood (Van Hees et al., 2022), future work seeks to determine the relationship between cumulative alcohol intake and cognitive outcomes, with a focus on how individual differences, such as sex and age of drinking-onset, modify this relationship. As a history of alcohol-drinking during adolescence/early adulthood induces microglial activation (Grifasi et al., 2019), as well as increases the expression of markers of Alzheimer's disease-related neuropathology (e.g., Salling et al., 2016; Hoffman et al., 2019), future work also seeks to relate alcohol-

induced cognitive anomalies, even those mild signs observed herein, to indices of neuropathology.

4.5. Conclusion

Herein we show that a 2-week history of binge-drinking by male and female, adult and adolescent, B6 mice induces relatively few signs of negative affect, some of which were sex-selective. Further, this binge-drinking history is sufficient to induce some signs of mild cognitive impairment in both adolescent- and adult- onset binge-drinkers that persist for greater than 1 month following the cessation of drinking.

Chapter 5:

Biochemical changes precede affective and cognitive anomalies in aging adult C57BL/6J mice with a prior history of adolescent alcohol binge-drinking

5.1. Introduction

Adolescence is a pivotal stage of behavioral and neurological development, characterized by significant maturation of brain regions governing emotion and cognition (Dorn, 2006; Spear, 2010; Paus, 2005). This developmental period is also characterized by an increased propensity for risky behaviors, including alcohol binge-drinking (Koob & Volkow, 2009; Novier et al., 2015; Spear & Varlinskaya, 2005). Although adults consume alcohol more frequently, adolescents between the ages of 12 to 20 years old account for approximately 11% of the total alcohol intake in the United States, predominately through binge-drinking (NM-IBIS, 2022). This pattern of drinking is typically defined as four or more drinks per occasion for women and five or more for men (NIAAA, 2023). Over 90% of adolescent alcohol intake occurs through binge-drinking episodes (Chung et al., 2018; NM-IBIS, 2022; SAMSHA, 2021). The neurotoxic impact of this binge pattern of consumption, marked by repeated heavy drinking episodes followed by periods of cessation, has been shown to exacerbate the potential for neurological harm (Duka et al., 2004) and is highly associated with the development of an alcohol use disorder (AUD) in adolescence (Addolorato et al., 2018).

Extant literature from human studies consistently indicates that a history of binge-drinking heightens susceptibility to mood disorders and cognitive deficits during alcohol withdrawal, with females experiencing these effects more severely than males (Cruz et al., 2023; Flores-Bonilla & Richardson, 2020; Peltier et al., 2019; Verplaetse et al., 2018). These observed sex differences are particularly troubling considering that women are more frequently diagnosed with depression and anxiety, disorders that are commonly exacerbated by their history of heavy alcohol consumption (Albert, 2015; Guinle & Sinha, 2020). The

cognitive repercussions of excessive alcohol in females are similarly disproportionate, with greater deficits in memory retention and spatial navigation capabilities (Mumenthaler et al., 1999). Moreover, women with a history of excessive alcohol consumption also show an increased incidence of cancer, accelerated liver problems, and exacerbated cardiovascular complications (Agabio et al., 2018; Connor et al., 2016; Jousilahti et al., 1999). The severity of these health concerns is even more alarming given the recent epidemiological trends revealing an 84% surge in excessive drinking among women, more than double the increase observed in men over the same period (Grant et al., 2017; Peltier et al., 2019). Additionally, there is a high co-occurrence of AUD and early-onset dementia, with AUD being a factor in nearly 60% of these dual diagnoses (Schwarzinger et al., 2018). This association is particularly concerning for women who have a twofold increased risk of developing alcohol-related dementias, including Alzheimer's Disease (AD), compared to men (Podcasy & Epperson, 2016), and is compounded by evidence that AUD among older female veterans is correlated with a more than threefold increase in dementia risk (Bahorik et al., 2021).

Evidently, an early onset of binge-drinking and biological sex serve as strong and important predictors for alcohol-related affective and cognitive disturbances, including Alzheimer's Disease and related dementias (ADRD). Although the biological underpinnings of these behavioral impairments are still not entirely understood, it is theorized that adolescent binge-drinking leads to neuronal alterations and the eventual degradation of key brain regions governing affective and cognitive processes (Carbia et al., 2017; Crego et al., 2009; Lees et al., 2020). Notably, adolescents with AUD exhibit disrupted neurodevelopmental trajectories, impacting the executive functions of the prefrontal cortex

(PFC), including decision-making and impulse control, with females showing more pronounced volumetric reductions within the PFC versus males (Medina et al., 2008). Adolescents with AUD also exhibit volumetric reductions of the hippocampus (HPC), a key region central to memory and learning (De Bellis et al., 2000; Nagel et al., 2005). The entorhinal cortex (EC) connects to the HPC via the perforant pathway (Ibáñez et al., 1995) and is important for memory consolidation and spatial navigation (Hyman et al., 1986). Notable cellular changes, including the reduction of neuronal nuclei size in layers II and III, have been observed in the EC of young and middle-aged humans (Ibáñez et al., 1995) and replicated in adolescent rats following a history of chronic alcohol use (Crews et al., 2000). Further, the EC is especially vulnerable in AD, sustaining the most extensive cortical damage (Van Hoesen et al., 1991), with animal studies suggesting that EC disruptions may serve as an early marker of ADRD pathology (Sipos et al., 2007). The amygdala (AMY), a core region for emotional processing and memory (McGaugh, 2004), has also been identified as an early site of AD-related pathological changes, including synaptic disruptions and volumetric reductions in humans (Gonzales-Rodriguez et al., 2023). In parallel, transgenic mouse models exhibit similar AD-related molecular markers such as amyloid pathology within the AMY, along with cognitive impairments apparent from as early as four months of age (Billings et al., 2005). Notably, these AD-related changes are most evident in the AMY of adult female 3xTg-AD transgenic mice following binge alcohol exposure during adolescence, in contrast to male mice, which, despite having a similar history of adolescent alcohol use, fail to show persistent increases in AD biomarkers in adulthood (Barnett et al., 2022). Such sex-dependent differences in ADRD-related biomarker prevalence emphasizes the AMY's vulnerability and its role in the development ADRD-related pathology.

Cellular and biochemical studies of rodent models of AUD support a cause-effect relationship between alcohol experience and perturbed emotionality and cognitive deficits (Barnett et al., 2022; Jimenez Chavez et al., 2022, 2020; Szumlinski et al., 2023). Our laboratory's previous work with C57BL6/J (B6) mice demonstrated that a two-week history of binge-drinking initiated in adolescence can elicit biochemical changes within several mesocorticolimbic regions (Lee et al., 2016, 2017), which manifest during protracted withdrawal as a spectrum of behavioral anomalies from hyperactivity to depression-like symptoms (Lee et al., 2017; Szumlinski et al., 2019; Jimenez Chavez et al., 2023). Moreover, both adolescent and adult female mice not only consume greater amounts of alcohol but also tend to exhibit more pronounced signs of alcohol-induced behavioral anomalies than males (Jimenez Chavez et al., 2020, 2023; Szumlinski et al., 2019). A history of repeated alcohol exposure is well-characterized to augment both pre- and postsynaptic indices of glutamate transmission throughout the brain (Brunett et al., 2016; Rao et al., 2015; Roberto & Varodayan, 2017) and alcohol-induced glutamate excitotoxicity is theorized to contribute significantly to alcohol-related neurodegeneration underpinning the loss of executive function, volitional control and cognitive decline (Brust, 2010; Peng et al., 2020). Consistent with this, young adult (~2.5 month-old) B6 mice with a prior 2-week history of adolescent binge-drinking exhibit increased protein expression of key glutamatergic signaling proteins, including Group 1 metabotropic glutamate receptors (mGlu1, mGlu5), ionotropic glutamate receptor subunits (AMPA and NMDA), the glutamate receptor-associated scaffolding proteins Homer 1b/c and Homer 2a/b, that correlate with heightened negative affect (Campbell et al., 2019; Lee et al., 2016, 2017, 2018a, 2018b; Szumlinski et al., 2023). Such

findings argue that a history of adolescent binge-drinking can produce latent effects in the brain and behavior that manifest later in adulthood (Alaux-Cantin et al., 2013; Barnett et al., 2022; Crews et al., 2016; Quoilin et al., 2012). However, our more recent study indicates that the brain and behavioral disturbances instigated by a 2-week history of adolescent binge-drinking may not persist throughout adulthood, as they were less apparent in adult mice tested ~ 4-months of age (Jimenez Chavez et al., 2020).

Building upon our previous research, the present study explored the biobehavioral effects of a more prolonged, one-month, history of binge-drinking that commences in early adolescence by tracking the course of affective and cognitive anomalies at three later developmental stages, 6-, 9-, and 12-months of age (i.e., from mature adulthood to middle age; Flurkey et al., 2007). Guided by our prior immunoblotting work (Lee et al., 2016, 2017a, 2017b, 2018a; Szumlinski et al., 2023) and evidence that a history of binge-drinking during mature adulthood is sufficient to elevate certain protein indices of ADRD-related neuropathology within the brain during early withdrawal (Jimenez Chavez et al., 2023), we examined for changes in glutamate receptor-related protein expression within the PFC, HPC, EC and AMY, as well as indices of ADRD-related neuropathology, including BACE isozyme (Fukumoto et al., 2004; Holsinger et al., 2002;) and phospho-tau expression (Fukumoto et al., 2002; Janelidze et al., 2020; Johnson & Stoothoff, 2004). We hypothesized that a 1-month long history of binge-drinking during adolescence and into young adulthood would accelerate the onset and progression of normal age-related cognitive and affective anomalies, particularly in female subjects. Secondly, we hypothesized that behavioral

anomalies would be associated with heightened indices of glutamatergic signaling and markers of neuropathology.

5.2 Materials and Methods

5.2.1 Subjects

Male and female C57BL/6J (B6) mice, PND21-25, were purchased from the Jackson Laboratory (Sacramento, CA) and allowed to acclimate to the colony room for 1 week prior to commencing drinking procedures (see below). Mice were housed in same-sex groups of four in standard polycarbonate cages on a ventilated rack in a climate- and humidity-controlled holding room. Cages were lined with sawdust bedding and contained nesting material and a plastic enrichment device in accordance with vivarium protocols. All mice were housed under a reverse light cycle (lights off: 1100 h; lights on: 2300 h), with food and water available *ad libitum* throughout the study. Mice arrived in cohorts of 48 (24 females and 24 males), with cohorts spaced approximately 1 month apart to accommodate drinking procedures. All experimental procedures were in compliance with The Guide for the Care and Use of Laboratory Animals (2014) and approved by the Institutional Animal Care and Use Committee of the University of California, Santa Barbara. A summary of the procedural time-line for this study is provided in **Figure 5.1**.

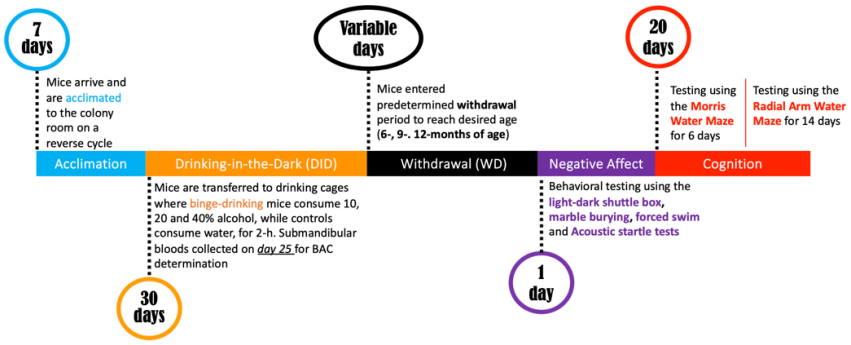


Figure 5.1. Illustration depicting of the procedural timeline of the experiments conducted in the current study.

5.2.2 Drinking-in-the-Dark (DID) Procedures

Half of the mice in each cohort (i.e., 12 males and 12 females) were subjected to 30 consecutive days of alcohol-drinking using a multi-bottle-choice DID procedure, beginning at approximately PND28-32. At 2-h after lights out (i.e., 1300 h), alcohol-drinking (EtOH) animals were transferred to individual drinking cages that were lined with sawdust bedding and topped with a wire lid, situated on a free-standing rack within the colony room. Mice were allowed to habituate to the drinking cage for 1-h, at which time, alcohol-drinking mice (EtOH) were allowed concurrent access to unadulterated ethanol 10, 20 and 40% (v/v) solutions in tap water (e.g., Cozzoli et al., 2012; Lee et al., 2016), with the location of sipper tubes randomized daily. Animals were allowed to drink for 2 h (1400 – 1600h). During the 1-h habituation and 2-h alcohol drinking periods, water control mice (H2O) underwent our simplified water drinking procedures in which daily handling and removal from the home cage were controlled for by placing H2O mice, with their cage mates, into a novel drinking cage on the same free-standing rack as the EtOH mice for 1-h and then presenting them with a single sipper tube containing water for the 2-h drinking period (e.g., Jimenez Chavez et al.,

2020; Lee et al., 2018; Szumlinski et al., 2019). At 16:00h, the sipper tubes were removed from the drinking cages and both the EtOH and H2O mice were then transferred back into their home cages.

For all cohorts, the alcohol-containing sipper tubes were weighed prior to, and immediately following, each 2-h drinking session to determine the volume consumed. The alcohol/water in the bottles was refreshed and all the mice were weighed every 3-4 days during the month-long drinking procedures. The recorded body weights of the mice were used to calculate alcohol intake on a g/kg body weight basis.

5.2.3 Blood Ethanol Concentrations

On the 25th drinking day, submandibular blood samples were collected from the alcohol-drinking mice only, immediately after the 2-h alcohol-drinking period and samples were stored at -200C until processing (7-10 days following collection). Headspace gas chromatography using a Shimadzu GC-2014 gas chromatography system (Shimadzu, Columbia, MD) was employed to analyze blood ethanol concentrations (BECs) as in recent reports (e.g., Jimenez Chavez et al. 2020, 2022, 2023). BECs were determined via the GC Solutions 2.10.00 software in samples diluted at 1:9 with non-bacteriostatic saline (50µl of sample). Toluene was used as the pre-solvent and the determination of ethanol from each sample was derived using the standard curve equation determined prior to analyses of the blood samples. A new standard curve was formulated for each cohort of blood samples to ensure maximal accuracy. After the ethanol peak area was determined, the peak area was

used to determine the ethanol concentration and subsequently the percent of ethanol in the blood.

5.2.4 Behavioral Test Battery for Negative Affect

To test the hypothesis that a 1-month history of binge-drinking during the period of adolescence into young adulthood might induce long-lasting changes in negative affect, a 1-day behavioral test battery for negative affect was conducted when the mice were 6, 9 or 12 months of age (respectively, 6M, 9M and 12M). As in our prior studies (e.g., Jimenez Chavez et al., 2020, 2023; Lee et al., 2015; Szumlinski et al., 2019), this behavioral test battery consisted of the light-dark shuttle-box, marble-burying, acoustic startle and forced swim test, which were run in series and mice remained in their home cages in the procedural room between paradigms. The behavioral testing equipment was cleaned in-between each use with Rescue Disinfectant Veterinary Wipes (Virox Animal Health, Oakville, ON, Canada). The details of each specific assay are provided below. Consistent with recent studies (Jimenez Chavez et al., 2020, 2023), males and females were tested for negative affect on separate days to minimize any pheromonal influences on affective behavior (Jimenez Chavez & Szumlinski, in press).

5.2.4.1. Light–Dark Shuttle Box

The light–dark shuttle-box was used to measure photophobia, with decreased activity in the light-side interpreted as reflecting an anxiety-like phenotype (Crawley, 1985; Gallo et al., 2014). Animals were placed into a polycarbonate box (46 cm long × 22 cm wide × 24 cm high) that was divided into two environments, one side is white with a clear lid and the other

side was black with a black lid (respectively, light versus dark side) that were accessible through a central divider with an opening. Testing commenced by placing the mice into the dark environment. The latency to enter the light side, total time spent in the light side and total number of light entries were recorded over a 5-min period using digital video cameras mounted above the test apparatus and ANYMaze software (Stoelting, Wood Dale, IL) by trained experimenters blind to the prior drinking histories of the mice.

5.2.4.2 Marble-Burying Test

The marble-burying test is particularly sensitive to the anxiogenic effects of alcohol withdrawal, based on our prior work with adolescent and young adult (i.e., 2-3 month-old) mice (e.g., Jimenez Chavez et al., 2020; Lee et al., 2015, 2016, 2017a,b; 2018a,b; Szumlinski et al., 2019). For this assay, mice were placed in a polycarbonate cage (12 cm × 8 cm × 6 cm), with 5-cm deep sawdust bedding on top of which 20 black marbles were arranged equidistantly. Mice were left undisturbed for a period of 20 min at which time, the number of marbles buried (i.e., 75% covered by bedding) was determined by an experimenter who was blind to the drinking history of the mice.

5.2.4.3 Acoustic Startle and Pre-Pulse Inhibition of the Acoustic Startle

The apparatus and procedures employed to assay the magnitude of acoustic startle and prepulse inhibition of acoustic startle were similar to those described previously by our group (e.g., Datko et al., 2017; Lominac et al., 2005; Szumlinski et al., 2005a). Six different trial types were presented: startle pulse (st110, 110 dB/40 msec), low prepulse stimulus given alone (st74, 74 dB/20 msec), high prepulse stimulus given alone (st90, 90 dB/20 msec), st74

or st90 given 100 msec before the onset of the startle pulse (pp74 and pp90, respectively) and no acoustic stimulus (i.e. only background noise was presented; st0). St100, st0, pp74 and pp90 trials were applied 10 times, st74 and st90 trials were applied five times, and all trials were given in random order. The average intertrial interval was 15 seconds (10–20seconds), and the background noise of each chamber was 70 dB. The data for startle amplitude were averaged across each of the stimulus trial types for statistical analyses of startle magnitude. The percent inhibition of the 110dB startle by the 74- and 90-dB prepulse intensities was also calculated for each animal.

5.2.4.4 Forced Swim Test

The forced swim test is a commonly employed assay for the reversal of passive coping behavior by anti-depressant treatments (Porsolt et al., 2001). Excessive swimming behavior in this assay can be reversed by pretreatment with anxiolytic medications (Lee et al., 2017b) and thus, has been used by our group as an additional measure of anxiety-like behavior during alcohol withdrawal (e.g., Jimenez Chavez et al., 2020, 2023; Lee et al., 2018a,b; Szumlinski et al., 2019). The swim "tank" consists of an 11-cm diameter cylindrical glass container, filled to 15 cm from the rim, with room temperature water. Mice are lowered into the tank and tested over a 6-min period during which AnyMaze^{TM} tracking software records the latency to first immobile episode, total time spent immobile, and the number of immobile episodes. Immobility is defined as the lack of vertical or horizontal displacement of the animal's center of gravity for at least 5-s. Upon the conclusion of this assay, animals were allowed to dry prior to being returned to their home cage and the holding room.

5.2.4.5 Morris Water Maze

The day following testing for negative affect, mice were assayed for spatial learning and memory using Morris water maze procedures akin to those published previously by our laboratory (e.g., Datko et al., 2017; Denning et al., 2024; Jimenez Chavez et al., 2023). The maze consisted of a stainless-steel circular tank (200 cm in diameter, 60 cm in height; filled with room temperature water to a depth of 40 cm), with salient intra-maze cues located on all four sides of the tank (star, square, sun and stripes). To ensure equivalent visual processing in all mice at the outset of each experiment, a "flag test" was first performed, in which the clear platform was placed in the tank in the NW quadrant with a patterned flag attached that extended 6 inches above the water. Over the course of the next 4 days, the clear platform (unflagged) remained in a fixed location in the NE quadrant (i.e., a quadrant distinct from that employed in the flag test). Each day, mice were trained four times a day (once at each compass point) to locate the hidden platform. During each trial, mice were randomly placed in the pool at one of the four compass points and swimming was recorded digitally by a video camera mounted on the ceiling directly above the pool (ANY-Maze, Stoelting). Training sessions were 2-min in duration and mice were tested in series at each compass release point. Mice unable to locate the platform during the allotted time were guided to the platform using forceps, where they remained for 30 sec. At 24 h after the last training trial, a 2-min memory probe test was performed in which the platform was removed from the pool and the amount of time taken by the mouse to swim toward the former platform location and the number of entries into the former platform location was recorded. The next day, a reversal training session was conducted in which the platform (unflagged) was situated in the SW quadrant (i.e., the quadrant opposite to that employed during the training phase of the

experiment). Again, mice were trained to locate the platform over 4, 2-min, sessions (one training trial for each compass point) to locate the repositioned platform (Denning et al., 2024; Jimenez Chavez et al., 2023).

5.2.4.6 Water Version of the Radial Arm Maze

Following the Morris maze testing, working and reference memory were determined using a water version of the radial arm maze with procedures similar to those employed in our prior studies (Denning et al., 2024; Jimenez Chavez et al., 2023). The maze consisted of 8 arms with clear, hidden, escape platforms at the ends of 4 of the arms. The start arm was the same for all the mice and remained constant throughout. Each mouse was assigned different platform locations that remained fixed throughout the experiment and the baited arms were semi-randomly assigned across subjects. A subject had 180 sec to locate a platform. If the mouse was unsuccessful at locating a platform in the allotted time, it was guided to the nearest available platform using forceps. Once a platform was found, the animal remained on it for 15 sec, and was then returned to an empty, heated, holding cage for 30 sec. During that time, the located platform was removed from the maze. The animal was then placed back into the start arm and allowed to locate another platform. Each day, this sequence of events repeated until the mouse located all four platforms. Thus, each mouse underwent four trials per day, with the working memory system taxed increasingly with each trial. As in the land version of this maze, animals have to avoid arms that never contained a reinforcer (reference memory) and enter only once into arms that contained a reinforcer (working memory). Day 1 was considered a training session because the animal had no previous experience in the maze. Days 2-7 were testing sessions and errors were quantified

for each day using the orthogonal measures of working and reference memory errors (Jarrard et al. 1984), as conducted previously by our group (Denning et al., 2024; Jimenez Chavez et al. 2023) and others (Bimonte et al. 2000). Working Memory Correct errors were the number of first and repeat entries into any arm from which a platform had been removed during that session. Reference Memory errors were the number of first entries into any arm that never contained a platform. Working Memory Incorrect errors were the number of repeat entries into an arm that never contained a platform in the past (thus, repeat entries into a reference memory arm).

5.2.4.7 Tissue Dissection and Immunoblotting

As recent immunoblotting studies indicated interactions between age, sex and a history of binge-drinking on the expression of glutamate receptor-related proteins, as well as protein indices of ADRD-related neuropathology, within the PFC and hippocampus of 6M and 18M B6 mice (Szumlinski et al., 2023), we determined whether a prior history of binge-drinking during early life could accelerate age-related changes in these proteins in a sex-dependent manner. For this, mice employed in the behavioral study were decapitated approximately 24 h following the last radial arm maze session. Brains were extracted and cooled on ice, then the brain was sectioned in 1 mm-thick coronal slices. The PFC was dissected out using blunt forceps, the EC and amygdala were dissected using an18-gauge needle and then both the ventral and dorsal hippocampus removed with blunt forceps and tissue from both hippocampal subregions combined into a single sample. Unfortunately, the hippocampal samples were accidentally subjected to over-heating, resulting in the

degradation of protein and could not be processed. Thus, only the data for the PFC, EC and amygdala are presented herein.

To index total NMDA receptor expression, we immunoblotted for the obligatory GluN1 subunit. We also immunoblotting for GluN2b expression as GluN2b is well-characterized to be highly alcohol-sensitive (e.g., Wills et al., 2017), is up-regulated in a number of brain regions in adult mice with a history of binge-drinking (Cozzoli et al., 2009, 2012, 2014, Lee et al., 2016, 2017a,b, 2018a,b; Szumlinski et al., 2023) and most relevant to this study, is upregulated in adult rodents following a history of adolescent alcohol exposure (Schwartzwedler et al., 2016). Likewise, both the mGlu1 and mGlu5 subtypes of mGluRs are also typically up-regulated in mice with a history of binge-drinking (Cozzoli et al., 2009, 2012, 2014; Szumlinski et al., 2023), to include adult mice with a history of binge-drinking during adolescence (Lee et al,. 2016, 2017a,b, 2018a,b). The signaling and localization of both NMDA and Group 1 mGluRs are regulated in brain by the Homer1b/c and Homer2a/b members of the Homer family of scaffolding proteins (Xiao et al,. 1998; Szumlinski et al., 2005b), of which Homer2a/b is highly alcohol-sensitive and gates the rewarding/reinforcing and sedative properties of alcohol (Szumlinski et al., 2008; Cozzoli et al., 2009, 2012, 2014, 2015), as well as the manifestation of negative affect during protracted withdrawal from adolescent binge-drinking (Lee et al., 2018c). Thus, we immunoblotted also for Group1 mGluRs and their Homer scaffolding proteins. As it was predicted that an upregulation of glutamate receptor expression would increase the activational state of our regions of interest, we examined for p(Tyr204) ERK1/2 expression as an index of cellular activity. A number of proteins currently serve as strong and reliable biomarkers of AD in human brain (Banning et al., 2021; Cheignon et al., 2018; Dodart et al., 2002; Hamley, 2012; Hersi et al., 2017; Perl,

2010) that can accumulate in brain during normal aging in both humans (Arriagada et al., 1992; Haroutunian et al., 1998; O'Brien et al., 2009; Troncoso et al., 1998) and induced by prior alcohol experience in laboratory rodents (Liu et al., 2022; Hoffman et al., 2019; Salling et al., 2016; Szumlinski et al., 2023). Thus, we assayed also for the following proteins: amyloid precursor proteins (APP), amyloid-β peptides (AB), hyper-phosphorylated tau proteins, and beta secretase (BACE).

The tissue homogenization and immunoblotting procedures employed in the present study were very similar to those detailed in our earlier reports (Chiu et al., 2021; Denning et al., 2024; Huerta Sanchez et al., 2023; Szumlinski et al., 2023). The following rabbit primary antibodies were used: mGlu5 (metabotropic glutamate receptor 5; 1:1000 dilution; Millipore; AB5675), GluN1 (NMDA receptor subunit 1; 1:500 dilution; Cell Signaling Technology; 5704S), Homer2a/b (1:500 dilution; Synaptic Systems; 160 203), p(Tyr204)ERK1/2 (1:750 dilution; R&D systems; AF1018), APP (1:1000 dilution; Millipore-Sigma; 07-667), amyloid beta (1:500 dilution; Abcam, ab180956), p(Ser396)-tau (1:750 dilution; Abcam; ab109390), and p(Thr217)-tau (1:500 dilution; Invitrogen, 44-744). The following mouse primary antibodies were also employed: mGlu1 (metabotropic glutamate receptor 1; 1:500 dilution; BD Biosciences; 610965), GluN2b (NMDA subunit 2b; 1:500 dilution; Invitrogen; MA1-2014), Homer1b/c (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-25271), ERK1/2 (1:1000 dilution; Invitrogen, MA5-15605), tau (1:750 dilution; Invitrogen, AHB0042) and BACE (1:500 dilution; Millipore Sigma; MAB5308). Note that as reported in our earlier study (Huerta Sanchez et al., 2023), our selected mGlu1 antibody failed to reliably detect the dimer form of the receptor on every immunoblot. As such, only the monomer form

of mGlu1 is reported herein. Calnexin expression was employed to control for protein loading and transfer using either a rabbit or mouse primary anti-calnexin antibody (for rabbit, 1:1000 dilution; Enzo Life Sciences; ADI-SPA-860; for mouse, 1:500 dilution; Invitrogen, MA5-31501). Following primary antibody incubation, the membranes were washed with phosphate-buffered saline with tween (PBST), incubated in either a goat anti-rabbit IRDye 800CW secondary antibody (1:10,000 dilution; Li-Cor; 925-3221) or a goat anti-mouse IRDye 680RD secondary antibody (1:10,000 dilution; Li-Cor; 925-68070), and imaged on an Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE, USA). Raw values for each band were measured, and first normalized to their corresponding calnexin signal and then to the average value of the water control for that particular age and sex (see more details below).

5.2.4.8 Data Analyses

Given the complexity of our experimental design, we opted to conduct separate statistical analyses for males and females to enhance the clarity and interpretability of our behavioral findings. For variables associated with negative affect, we employed an Age (6, 9 and 12M) x Drinking History (H2O vs. EtOH) univariate Analysis of Variance (ANOVA), with Age and Drinking History as between-subject factors. Data from the acoustic startle test were analyzed using an Age x Drinking History x Stimulus ANOVA, with repeated measures on the Stimulus factor (4 levels). Data for prepulse inhibition of acoustic startle were analyzed using an Age x Drinking History x Prepulse ANOVA, with repeated measures on the Prepulse factor (2 levels). Data from the maze tests were examined using an Age x Drinking History ANOVA, with Day or Trial as a repeated measure, when appropriate. For

the immunoblotting data, we employed a Sex x Drinking History ANOVA. As this study employed 12 experimental conditions, immunoblotting procedures were performed independently for 6M, 9M and 12M mice, separately for both sexes. For each individual gel, results were normalized separately by sex in relation to the average of the control group (i.e. water-drinking mice). This approach yielded interaction effects that mirrored observations related to the Sex factor. Thus, we report on the interaction effect (Sex x Drinking History ANOVA), as well as the main effect of Drinking History. A complete set of statistical outcomes, including both significant and non-significant findings, is presented in **Tables 1 – 3**.

In cases where significant interactions were detected, we conducted simple main effect tests with Least Significant Difference (LSD) adjustments. Conversely, when significant main effects (> 2 levels) were observed without an interaction, LSD post hoc tests were employed to clarify group differences. The F-statistic, p-values, and partial eta squared values were reported for all statistical evaluations, with a pre-set alpha level of 0.05 for significance. Greenhouse–Geisser corrections were applied where sphericity was violated, and outliers were initially addressed using the ±1.5 × IQR rule. However, in cases where the initial method significantly reduced the sample size, the more lenient ±3 × IQR rule was implemented for the most extreme outliers. All statistical analyses were conducted using IBM SPSS Statistics (version 27.0 for Macintosh), Jamovi (version 2.3.21.0 for Macintosh), and the resulting graphs were produced with GraphPad Prism (version 10.2.2 for Macintosh).

5.3 Results

5.3.1 Alcohol Intake and BACs

A univariate Sex x Age ANOVA was conducted to determine group differences in the amount of alcohol consumed during the 30-day drinking period. As shown in **Figure 5.2A**, no significant Sex x Age interaction was detected [ANOVA: $p = 0.688$, $\eta 2 = .011$], however, significant main effects were observed: a Sex effect [$F(1,65) = 19.65$, $p < 0.001$, $\eta 2 = .232$] indicated higher alcohol consumption in female mice compared to male mice, and an Age effect [$F(2,65) = 7.26$, $p = 0.001$, $\eta 2 = .183$] demonstrated that 9M mice consumed less alcohol compared to 6M ($p = 0.001$) and 12M mice ($p = 0.003$), with no significant differences in intake between 6M and 12M mice ($p = 0.718$).

Despite these differences in intake, blood alcohol concentrations (BAC) measured on day 25 of drinking did not show an Age effect or interaction (**Figure 5.2B**; all p's > 0.338). Consistent with our intake data, a significant Sex effect [$F(1,65) = 4.41$, $p = 0.040$, $\eta^2 = .064$] detected that females showed higher BAC levels than males. Furthermore, a positive correlation was confirmed between BAC levels and alcohol intake on day 25 (**Figure 5.2C**; $r = .45$, $p < 0.001$), indicating that intake levels reliably predicted BAC irrespective of age, suggesting that age-related variations in consumption did not translate into differences in BAC.

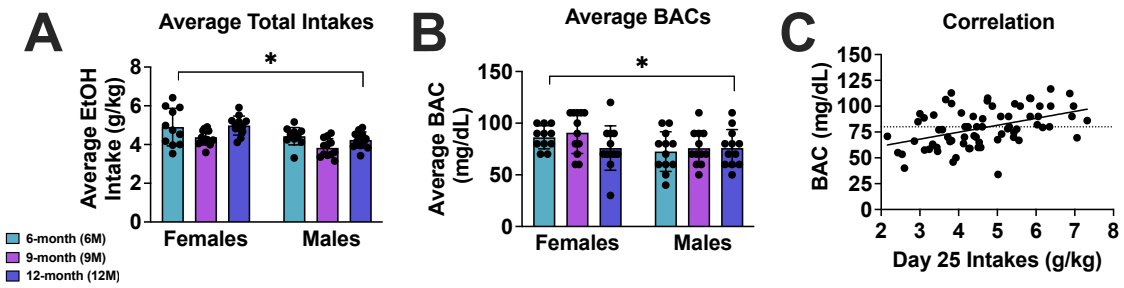


Figure 5.2. Depiction of the Sex and Age differences in alcohol intake and corresponding BAC levels. (**A**) Female mice consumed alcohol over the 30-day drinking period compared to males, with no significant interaction between sex and age. (**B**) BAC levels measured on Day 25, with females displaying higher BACs than males, reflecting the sex-specific intake patterns seen with total intake. (**C**) Scatter plot demonstrating a positive correlation between alcohol intake and BAC on Day 25. Figures show means ± SEMs. *p < 0.05, Female vs. Male.

Behavioral Measures

For transparency, the data for all groups are presented graphically, separately for females and males, with additional main effects or interactions also depicted to facilitate visualization of significant group differences. Throughout, we report both the results from the general linear model and estimates of effect sizes (η^2).

5.3.2 Light Dark Box Shuttle Test

An Age x Drinking History univariate ANOVA was conducted to determine group differences in the time taken to enter the light side of the light dark box for the first time. Results revealed no significant differences for female [ANOVA: $p = 0.746$, $\eta^2 = .010$, all other p's > 0.426; **Figure 5.3A**] or male mice [ANOVA: $p = 0.792$, $\eta^2 = 0.007$; all other p's > 0.112; **Figure 5.3B**]. While no significant Age x Drinking History interaction was observed for the total time spent by female mice in the light side of the light dark box [ANOVA: $p = 0.753$, $\eta^2 = 0.009$; **Figure 5.3C**], a significant main Age effect was found [$F(2,62) = 4.26$, $p = 0.019$, $\eta^2 = 0.121$; **Figure 5.3D**], and tests for multiple comparisons

indicated more time spent by 6M females when compared to both the 9M ($p = 0.019$) and 12M ($p = 0.008$) females. In contrast, no significant interaction or main effects were observed for the male mice [ANOVA; $p = 0.778$, $\eta^2 = 0.008$, all other p's > 0.503; **Figure 5.3E**]. As shown in **Figure 5.3F,** no significant Age x Drinking History interaction was found for the number of entries by female mice into the light side of the light dark box [ANOVA: $p = 0.528$, $\eta^2 = 0.020$]. However, significant main effects of Age [$F(2,62) = 10.37$, $p < 0.001$, $\eta^2 = .251$] and Drinking History [$F(1,62 = 5.57$, $p = 0.021$, $\eta^2 = 0.082$] were observed. For the main effect of Age in female mice (**Figure 5.3G**), subsequent tests for multiple comparisons revealed that 6M female mice exhibited a greater number of entries into the light side, compared to the 9M females ($p = 0.014$) and both the 6M and 9M females made more entries to the light side than the 12M females (6M vs. 12M: $p < 0.001$; 9M vs. 12M: $p = 0.047$). For the main effect of Drinking History in females (**Figure 5.3H**), binge-drinking females made less entries into the light side relative to their water-drinking counterparts. In contrast, no significant interaction or main effects were detected for the male mice [ANOVA: $p = 0.216$, $\eta^2 = .046$, all other p's > 0.358; **Figure 5.3I**].

5.3.3 Marble Burying Test

While no significant Age X Drinking History interaction was observed for the number of marbles buried by female mice [ANOVA: $p = 0.299$, $\eta^2 = 0.038$; **Figure 5.3J**], a significant main Age effect was found [$F(2,62) = 25.62$, $p < 0.001$, $\eta^2 = 0.452$; **Figure 5.3K**]. Post-hoc tests determined that 9M females buried more marbles than both the 6M ($p < 0.001$) and 12M ($p < 0.001$) female mice, with no significant differences between the 6M and 12M females ($p = 0.061$). A trend for an interaction was observed for the number of marbles

buried by males [$F(2,66) = 2.89$, $p = 0.063$, $\eta^2 = 0.080$; **Figure 5.3L**], with pairwise comparisons indicating that 9M male binge-drinking mice buried more marbles than their age-matched water-drinking counterparts ($p = 0.001$). No water-alcohol differences were observed for the 6M ($p = 0.096$) or 12M ($p = 0.355$) males.

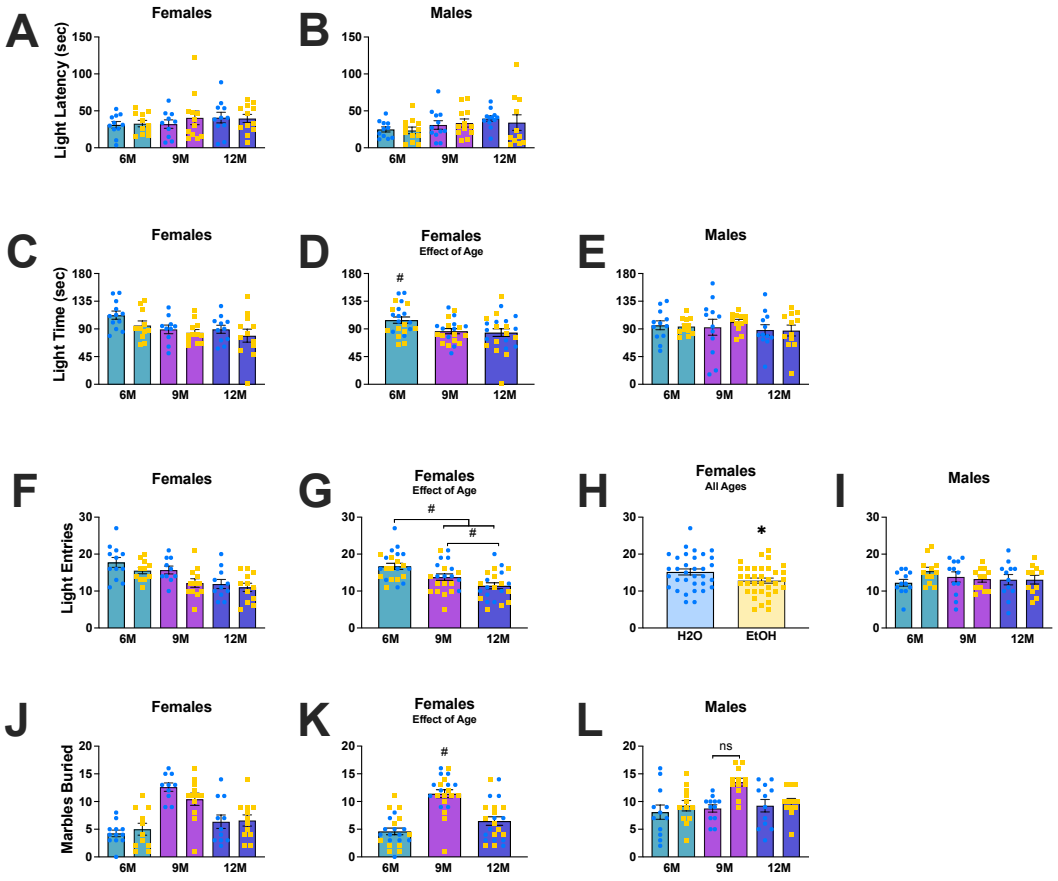


Figure 5.3. Depiction of the Age by Drinking History ANOVA results for the observed behavior in the light dark box shuttle and marble-burying tests for female and male mice.

Interpretation of the results observed for the latency to enter the light side of the light dark shuttle box test, where no significant interactions or main effects were observed for either the (**A**) female or (**B**) male mice. (**C**) Results for the total time spent in the light side failed to indicate a signficant interaction for the female mice, however, (**D**) a significant main effect of Age showed that 6M females spent significantly more time in the light side relative to the female mice in the other age groups. (**E**) No significant interaction or main effects were observed for the male mice in the total time spent in the light side. (**F**) Results for the total time spent in the light side failed to indicate a signficant interaction for the female mice, however, (**G**) a significant main effect of Age showed that the 12M females had fewer entries to the light side relative to the younger 6M and 9M mice and the 6M mice had the most

entries to the light side relative to both the 9M and 12M mice. Additionally, (**H**) a significant main effect of Drinking History showed that binge-drinking females made more entries to the light side when compared the water-drinking females. (**I**) There were no significant main effects or interactions observed in the total number of entries to the light side for male mice. Regarding the number of marbles buried in the marble-burying test, (**J**) no significant Age by Drinking History interaction was observed for the female mice; however, (**K**) a significant main effect of Age was found, indicating that 9M female mice buried more marbles compared to both 6M and 12M female mice. (**L**) For the male mice, an observable trend was detected, suggesting that at 9M, the binge-drinking mice buried more marbles than the water control mice, however this was not statistically significant. Figures show means ± SEMs. *p < 0.05, EtOH vs. H20; #p < 0.05, age difference, (ns)p > 0.05.

5.3.4 Forced Swim Test

No significant Age x Drinking History interaction or main effects were observed for the female mice regarding the latency to first float in the forced swim test [ANOVA: $p = 0.166$, $\eta^2 = 0.056$, all other p's > 0.149; **Figure 5.4A**]. While no significant interaction was observed for this variable in male mice [ANOVA: $p = 0.276$, $\eta^2 = 0.039$; **Figure 5.4B**], a significant main Age effect was detected [$F(2,65) = 3.88$, $p = 0.026$, $\eta^2 = 0.107$; **Figure 5.4C**]. Tests for multiple comparisons indicated that 6M males exhibited a shorter latency to first float than the 12M males ($p = 0.008$), with no other significant age differences noted [6M vs. 9M: $p = 0.103$; 9M vs. 12M: $p = 0.284$]. Although no significant Age x Drinking History interaction was observed for the female mice in the time spent immobile during the forced swim test [ANOVA: $p = 0.284$, $\eta^2 = 0.040$; **Figure 5.4D**], a significant main Drinking History effect was found [$F(2,61) = 5.25$, $p = 0.025$, $\eta^2 = 0.079$; **Figure 5.4E**], with binge-drinking females spending more time immobile than their water-drinking controls. In contrast, no significant interaction or main effects were observed for the male mice [ANOVA: $p = 0.212$, $\eta^2 = 0.047$, all other p's > 0.183; **Figure 5.4F**]. Further, no significant interaction or main effects of Age or Drinking History were detected for the number of immobile episodes exhibited by female [ANOVA: $p = 0.314$, $\eta^2 = .037$, all other p's >

0.080; **Figure 5.4G**] or male mice in the forced swim test [ANOVA: $p = 0.503$, $\eta^2 = 0.021$, all other p's > 0.117; **Figure 5.4H**].

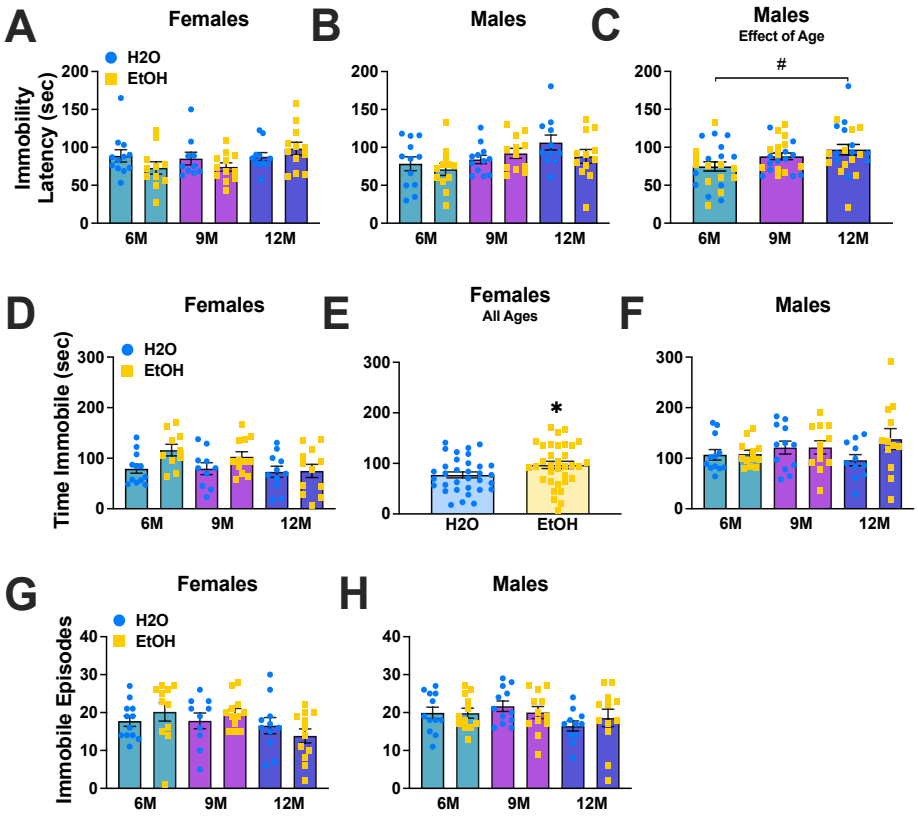


Figure 5.4. Depiction of the Age by Drinking History ANOVA results for the observed behavior in the forced swim test for female and male mice. For both female (**A**) and male (**B**) mice, no significant interaction was detected. However, (**C**) a significant main effect of Age was observed for the male mice showing that 6M mice showed a shorter latency to immobility relative to the 12M mice. (**D**) No significant interactions or main effects were observed for the total time spent immobile in the female mice, however, (**E**) a significant main effect of Drinking History was found, with binge-drinking females of all age groups spending more time immobile relative to water-drinking counterparts. Conversely, (**F**) no significant interactions or main effects were observed for the time spent immobile in male mice. Finally, no significant interaction or main effects were detected for the total number of immobile episodes in both female (**G**) and male (**H**) mice. Figures show means ± SEMs. *p < 0.05, EtOH vs. H20; #p < 0.05, age difference

5.3.5 Acoustic Startle and Prepulse Inhibition

An Age X Drinking History X Stimulus ANOVA indicated no group differences in the startle amplitude in response to the different acoustic stimuli (0-110 dB) for female mice [ANOVA: $p = 0.938$, $\eta^2 = 0.007$; Stimulus effect: $F(1.95, 115.28) = 64.01$, $p < 0.001$, $\eta^2 = .520$; **Figures 5.5A-C**]. Although the 3-way interaction was not significant in males [ANOVA: $p = 0.795$, $\eta^2 = 0.007$; **Figures 5.5D-F**], an Age X Stimulus interaction was detected [$F(3.49, 104.73) = 2.67$, $p = 0.043$, $\eta^2 = .082$; **Figure 5.5G**]. Post-hoc analyses revealed that when no acoustic stimulus was present (st 0 or 0 dB), the 12M mice exhibited less activity in the startle chamber, compared to both the 6M ($p = 0.015$) and 9M ($p = 0.008$) males. However, at the 90 dB stimulus (st90), the startle amplitude of 9M males was higher than that of 6M males ($p = 0.007$) but comparable to the 12M males ($p = 0.314$).

Having established comparable hearing in both female and male mice across the three ages (**Figure 5.5A-G**), mice were assayed for prepulse inhibition of acoustic startle. An Age x Drinking History x Prepulse Stimulus ANOVA failed to indicate any group differences in the percent inhibition by either the 74dB or 90dB pre-pulses [Female ANOVA: $p = 0.244$, $\eta^2 = .045$; PPI effect: $F(1, 66) = 245.63$, $p < 0.001$, $\eta^2 = 0.801$; **Figures 5.5H-J;** Male ANOVA: $p = 0.991$, $\eta^2 = 0.000$; PPI effect: $F(1,66) = 207.14$, $p < 0.001$, $\eta^2 = 0.758$; **Figures 5.5K-M**].

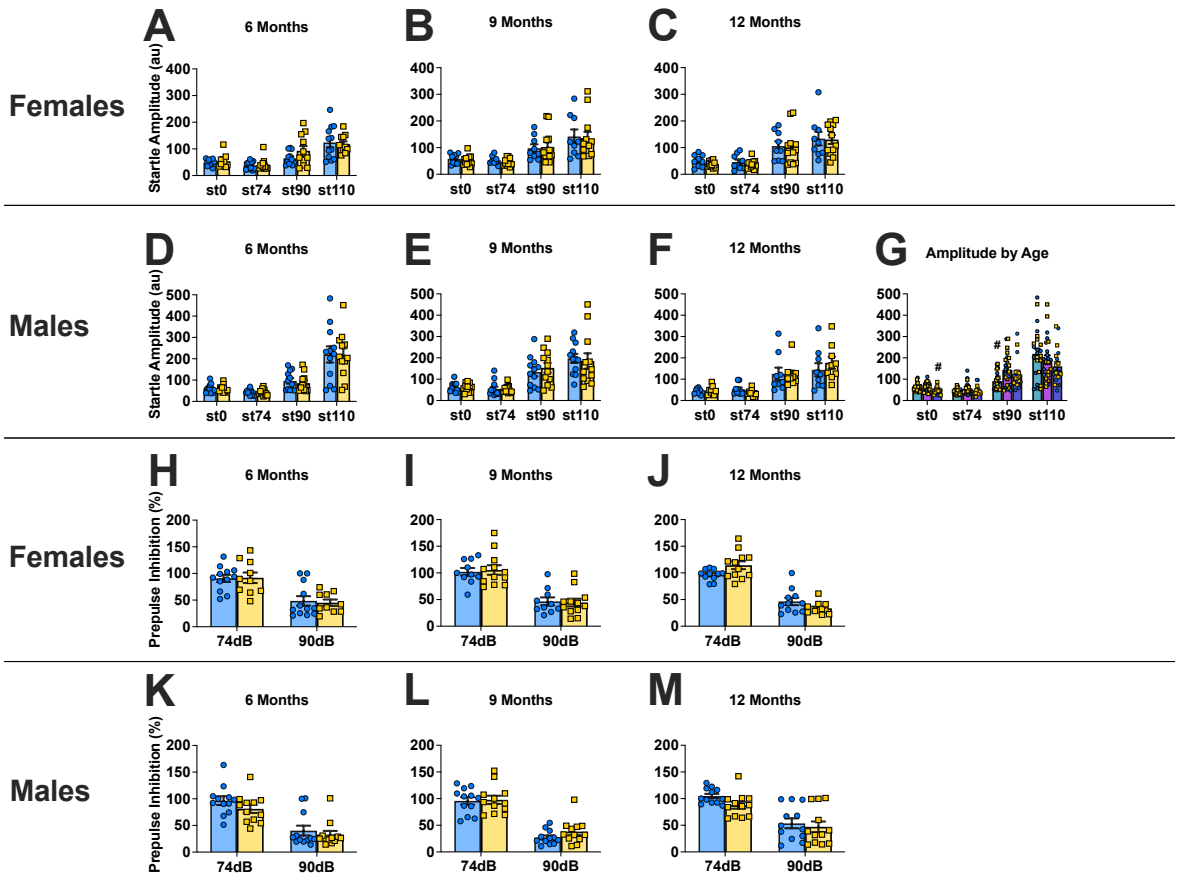


Figure 5.5. Depiction of the results for the observed behavior in the acoustic startle test for female and male mice. In the startle amplitude response to varying acoustic stimuli, results indicated no significant Age x Drinking History x Stimulus interaction for the (**A-C**) female mice or the (**D-F**) male mice. However, (**G**) a significant Age x Stimulus interaction was observed in male mice revealing lower activity at 0 dB in 12M compared to 6M and 9M males, and a higher startle amplitude at 90 dB in 9M compared to 6M. Similarly, no significant Age x Drinking History x Prepulse Stimulus interaction was noted for the (**H-J**) female or (**K-M**) male mice.

5.3.6 Morris Water Maze

5.3.6.1 Flag Test

An Age x Drinking History ANOVA failed to detect any significant interaction for the latency of female mice to locate the flagged platform [ANOVA: $p = 0.438$, $\eta^2 = 0.026$;

Figure 5.6A]. However, a significant main Age effect was observed [$F(2,62) = 3.75$, $p = 0.029$, $\eta^2 = 0.108$; **Figure 5.6B**] wherein 6M female mice located the visible platform more quickly than 12M female mice ($p = 0.009$), while no other age differences were evident (6M vs. 9M: $p = 0.093$; 9M vs 12M: $p = 0.338$). In contrast, no significant interactions of main effects were observed for male mice [ANOVA: $p = 0.721$, $\eta^2 = 0.010$, all other p's > 0.078; **Figure 5.6C**].

5.3.6.2 Maze Acquisition

An analysis of the average latency to locate the hidden platform during Morris maze acquisition indicated no significant 3-way interaction for the female mice [ANOVA: $p = 0.383$, $\eta^2 = 0.033$; **Figure 5.6D-F**]. However, a significant Day x Age interaction was found [$F(3.34, 101.95) = 5.16$, $p = 0.002$, $\eta^2 = 0.145$], which was then analyzed across the Day factor. As illustrated in **Figure 5.6G**, 12M females required significant more time to locate the platform than their 6M counterparts on Days 1 and 3 of training (Day 1: $p = 0.004$; Day 3: $p = 0.022$), but no other significant age-related differences were apparent in female subjects (p's > 0.062). Similarly, no significant 3-way interaction was observed for the latency of males to locate the hidden platform [ANOVA: $p = 0.344$, $\eta^2 = 0.036$; **Figures 5.6H-J**], but a significant Day x Age interaction was detected [$F(3.39, 103.49 = 3.05$, $p = 0.026$, $\eta^2 = 0.091$; **Figure 5.6K**]. This interaction was also deconstructed along the Day factor and found that on Day 3 of training, 6M males required more time to reach the platform than the 9M ($p = < 0.001$) and 12M ($p < 0.001$) male mice. No other significant age-related differences were apparent in male mice (p's > 0.083).

5.3.6.3 Probe Test

An Age x Drinking History ANOVA failed to detect a significant interaction or main effects for the time taken by female mice to first enter the former location of the platform during the Probe Test [ANOVA: $p = 0.952$, $\eta^2 = 0.002$, all other p's > 0.326; **Figure 5.6L**]. While no significant interaction was also detected for this variable in males [ANOVA: $p = 0.441$, $\eta^2 = 0.026$; **Figure 5.6M**], a significant main effect of Age was observed [$F(2,63) = 4.45$, $p = 0.016$, $\eta^2 = 0.124$]. As shown in **Figure 5.6N**, post-hoc analyses revealed that 9M males had a longer latency to re-enter the former location compared with the 6M ($p = 0.006$) and 12M males ($p = 0.020$). No differences were observed between 6M and 12M males ($p = 0.592$). A significant Age x Drinking History interaction was observed for the number of times female mice entered into the platform's former location in the maze during the 2-min probe test [$F(2,62) = 3.79$, $p = 0.028$, $\eta^2 = 0.109$; **Figure 5.6O**], prompting an analysis across the Age factor to identify differences between alcohol and water-drinking mice. Pairwise comparisons revealed that 9M binge-drinking females made fewer entries to the platform's former location than their water-drinking counterparts, while no significant differences between alcohol- and water-drinking mice were found for 6M ($p = 0.857$) or 12M ($p = 0.209$) females. In contrast to females, no significant interaction was detected for the number of entries into the former platform location by male mice [ANOVA: $p = 0.111$, $\eta^2 = 0.065$; **Figure 5.6P**], although a significant main Age effect was observed [$F(2, 66) = 24.88$, $p < 0.001$, $\eta^2 = 0.430$; **Figure 5.6Q**] that reflected more entries into the platform's former location by 12M males, compared to both the 6M ($p < 0.001$) and 9M ($p < 0.001$) male mice, with no difference observed between the 6M and 9M males ($p = 0.316$).

5.3.6.4 Reversal Learning

For both female (**Figures 5.6R-T**) and male (**Figures 5.6U-W**) mice, no significant Trial x Age x Drinking History interactions were found regarding the time taken to locate the repositioned platform during the test for reversal learning in the Morris Maze [Female ANOVA: $p = 0.688$, $\eta^2 = 0.019$; Trial effect: $F(1.81, 99.50) = 31.18$ $p < 0.001$, $\eta^2 = 0.362$; Male ANOVA: $p = 0.140$, $\eta^2 = 0.059$; Trial effect: [$F(1.43, 85.73) = 63.69$, $p < 0.001$, $\eta^2 = 0.515$].

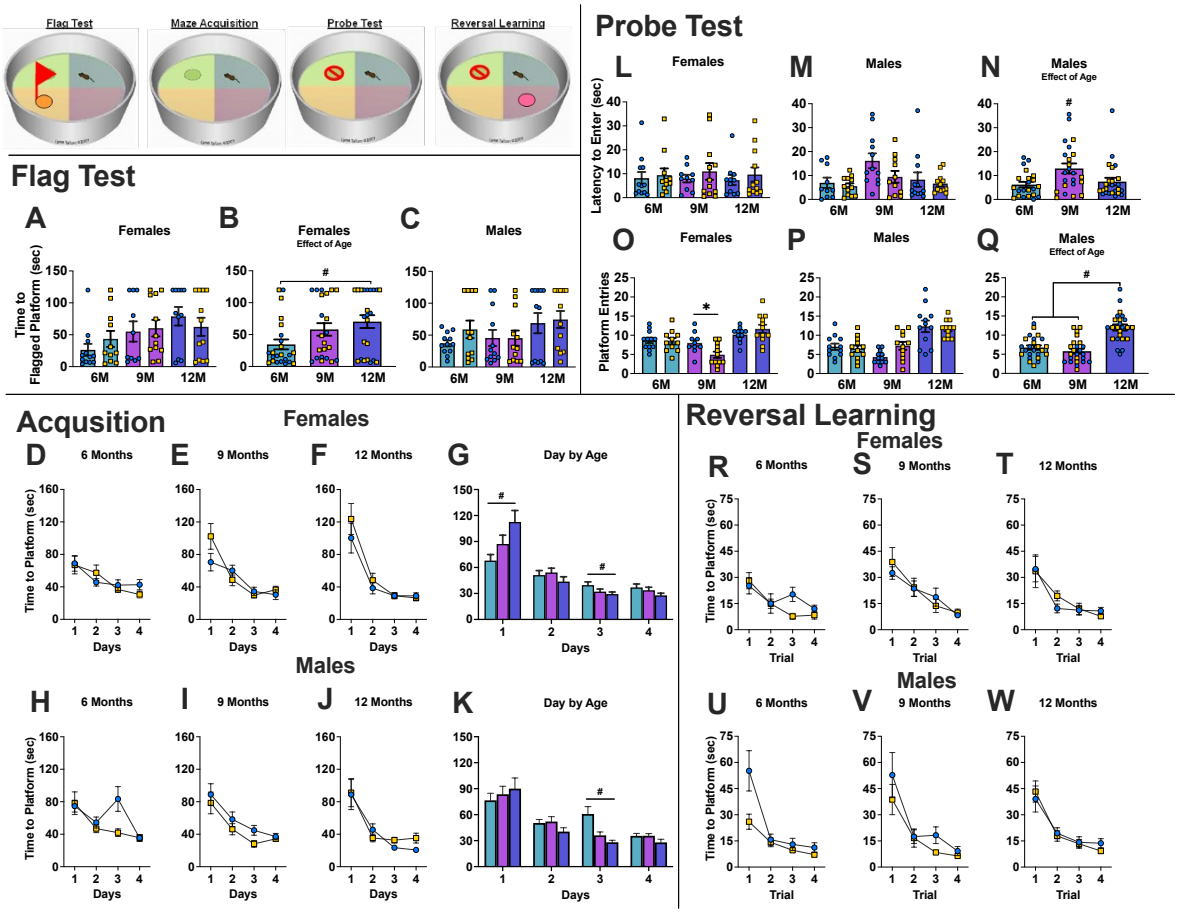


Figure 5.6. Depiction of the results for the observed behavior in the Morris water maze for female and male mice. In the flag test, (**A**) no significant Age by Drinking History ANOVA interaction was observed for the female mice, however, (**B**) a significant main effect

of Age found that 6M females required less time to locate the visible platformed relative to the older 12M female mice. In contrast, (**C**) no significant interaction or main effects were detected for the male mice. Depiction of the mixed-model Day by Age by Drinking History ANOVA results for the observed behavior during the acquisition training of Morris water maze for female and male mice. Mixed-model Day x Age x Drinking History ANOVA for the acquisition training showed (**D-F**) no group differences were observed for the average time taken to locate the hidden platform in (**H-J**) males. However, a significant Day x Age interaction was detected for both (**G**) females and (**K**) males, indicating a consistent decrease in the required time to locate the platform across the four days of training in all age groups. No drinking group differences were detected for the latency to enter the platform's former location in the NE quadrant during the probe test in (**L**) females or (**M**) males. However, (**N**) a significant main effect of Age found that 9M males exhibited a longer latency to enter the NE quadrant when compared to the 6M and 12M males. (**O**) A significant Age by Drinking History interaction was observed for the female mice in the number of entries to the NE quadrant, where 9M binge-drinking females had fewer entries to the NE quadrant relative to the 9M water-drinking females. (**P**) In contrast, no significant interaction was observed for the male mice, however, (**Q**) a significant main effect of age demonstrated that 12M males made more entries to the NE quadrant relative to both the 6M and 9M males. In the reversal test, neither the (**R-T**) female or (**U-W**) male mice exhibited significant interactions in the time taken to locate the repositioned platform. Figures show means ± SEMs. #$p < 0.05$, age difference

5.3.7. Radial Arm Maze

5.3.7.1 Reference Memory Errors

A very strong statistical trend for a Day x Age x Drinking History interaction was observed for the number of reference memory errors committed by female mice during radial arm maze testing [$F(7.76, 240.63) = 1.98$, $p = 0.051$, $\eta^2 = 0.060$; **Figure 5.7A-C**]. Deconstruction of the 3-way interaction along the Age factor failed to detect significant Day x Drinking History interactions or main effects for the 6M (ANOVA: $p = 0.216$, $\eta^2 = 0.066$; all other p's > 0.164; **Figure 5.7A**) or the 12M females (ANOVA: $p = 0.908$, $\eta^2 = 0.011$; all other p's > 0.127; **Figure 5.7C**]. However, a significant Day x Drinking History interaction was observed for the 9M females [$F(5,100) = 3.19$, $p = 0.010$, $\eta^2 = 0.138$; **Figure 5.7B**] that reflected more reference errors on days 2, 6 and 7 by binge-drinking females, compared to

their water-drinking counterparts (p's < 0.032; all other p's > 0.057). Similarly, a significant Day x Age x Drinking History interaction was observed for the male mice [$F(10,330) = 2.36$, $p = 0.010$, $\eta^2 = 0.067$; **Figure 5.7D-E**]. In the case of males, a significant Day x Drinking interaction was identified in 6M mice [$F(5,110) = 2.164$, $p = 0.063$, $\eta^2 = 0.090$; **Figure 5.7D**], that reflected more reference memory errors by binge-drinking males than their water-drinking counterparts on day 2 ($p = 0.020$; all other p's > 0.080). Although no significant Day x Drinking History interaction or main effects were observed for the 9M male mice [ANOVA: $p = 0.395$, $\eta^2 = 0.045$; all other p's > 0.365; **Figure 5.7E**], a significant Day x Drinking History ANOVA was observed for the 12M males [$F(5,110) = 2.34$, $p = 0.046$, $\eta^2 = 0.096$; **Figure 5.7F**], that reflected fewer reference memory errors by binge-drinking versus water-drinking males on day 5 ($p = 0.040$; all other p's > 0.078).

5.3.7.2 Working Memory Correct Errors

A significant Day x Age x Drinking History interaction was detected for the number of working memory correct errors committed by female mice [$F(10,310) = 2.32$, $p = 0.012$, $\eta^2 = 0.070$; **Figure 5.7G-I**]. Deconstruction of this interaction along the Age factor did not detect a significant Day x Drinking interaction for 6M females [ANOVA: $p = 0.372$, $\eta^2 = 0.049$; Day effect: $F(5,105) = 2.45$, $p = 0.039$, $\eta^2 = 0.104$; **Figure 5.7G**]. A trend for a Day x Drinking History interaction was observed for the 9M females [$F(5,100) = 2.17$, $p = 0.063$, $\eta^2 = 0.098$; **Figure 5.7H**]. A significant Day x Drinking History interaction was observed for the 12M female mice [$F(5, 105) = 2.85$, $p = 0.019$, $\eta^2 = 0.120$; **Figure 5.7I**], which reflected more errors by binge-drinking females than water controls on day 3 (Day 3: $p = 0.014$; other days, p's > 0.069). In contrast to females, no significant Day x Age x Drinking History

interactions or main effects were found for the male mice [ANOVA: $p = 0.770$, $\eta^2 = 0.019$; all other p's > 0.156, **Figures 5.7J-L**].

5.3.7.3 Working Memory Incorrect Errors

A significant Day x Age x Drinking History interaction was found for the number of working memory incorrect errors committed by the female mice [$F(10,310) = 2.00$, $p = 0.033$, $\eta^2 = 0.061$; **Figure 5.7M-O**]. Deconstruction of the interaction along the Age factor did not detect a significant Day x Drinking History interaction for the 6M females [ANOVA: $p = 0.994$, $\eta^2 = 0.004$; Day effect: $F(5,105) = 2.26$, $p = 0.054$, $\eta^2 = 0.097$; **Figure 5.7M**] or the 9M females (ANOVA: $p = 0.150$, $\eta^2 = 0.083$; all other p's > 0.183; **Figure 5.7N**). In contrast, a significant Day x Drinking History interaction was identified for the 12M females [$F(5,105) = 2.64$, $p = 0.028$, $\eta^2 = 0.112$; **Figure 5.7O**] that reflected more working memory incorrect errors committed by binge-drinking versus water controls on day 3 (day 3: $p = 0.040$; other days: p's > 0.131). In contrast to the female mice, no significant 3-way interaction was detected for the number of working memory incorrect errors committed by male mice [ANOVA: $p = 0.177$, $\eta^2 = 0.041$; **Figure 5.7P-R**), although a significant Day x Age interaction was observed [$F(10,330) = 2.66$, $p = 0.004$, $\eta^2 = 0.075$]. As shown in **Figure 5.7S,** test for simple main effects revealed that on days 2 and 4, 6M male mice committed more working memory incorrect errors compared to their 9M counterparts (Day 2: $p = 0.049$; Day 4: $p = 0.011$). On day 3, the 12M males had more working memory errors relative to both the 6M ($p = 0.034$) and 9M ($p = 0.004$) male mice However, on day 7, 9M males exhibited a greater number of working memory incorrect errors than the 12M males ($p = 0.025$).

5.3.7.4 Time Taken to Complete the Radial Arm Maze

A significant Day x Age x Drinking History interaction was detected for the time taken by female mice to complete the radial arm maze [$F(10,310) = 2.75$, $p = 0.003$, $\eta^2 = 0.081$; **Figure 5.7T-V**]. Deconstruction of this interaction along the Age factor failed to indicate any interaction or main Drinking History effect for the 6M mice (ANOVA: $p = 0.447$, $\eta^2 = .044$; Day effect: $F(5,105) = 9.91$, $p < 0.001$, $\eta^2 = 0.321$; **Figure 5.7T**). A significant Day x Drinking History interaction was detected for the 9M females [$F(3.28, 65.50) = 3.51$, $p = 0.017$, $\eta^2 = 0.149$; **Figure 5.7U**], which reflected a longer time taken by 9M females to complete the maze on day 4 of training ($p = 0.005$; all other p's > 0.066). A significant Day x Drinking History interaction was also detected for 12M females [$F(3.59, 75.37) = 2.88$, $p = 0.033$, $\eta^2 = 0.121$; **Figure 5.7V**], that reflected a longer time taken by binge-drinking versus water controls on day 3 of training ($p = 0.013$; all other p's > 0.122). Akin to females, a significant Day x Age x Drinking History interaction was detected for the male mice [$F(10,330) = 2.82$, $p = 0.002$, $\eta^2 = 0.079$; **Figure 5.7-W-Y**]. Deconstruction of this interaction along the Age factor detected a significant Day x Drinking History interaction for the 6M males [$F(5,110) = 4.53$, $p = 0.001$, $\eta^2 = 0.171$; **Figure 5.7W**], which reflected less time by binge-drinking mice to complete the maze compared to their water-drinking counterparts on days 2, 6 and 7 (p's < 0.046; all other p's > 0.162). No significant interaction or main effects were found for the 9M male mice [ANOVA: $p = 0.195$, $\eta^2 = 0.064$; other p's > 0.681; **Figure 5.7X**] and no significant interaction or main Drinking History effect was observed for the 12M males [ANOVA: $p = 0.822$, $\eta^2 = 0.019$; Day effect: $F(5,110) = 2.59$, $p = 0.029$, $\eta^2 = 0.105$; **Figure 5.7Y**].

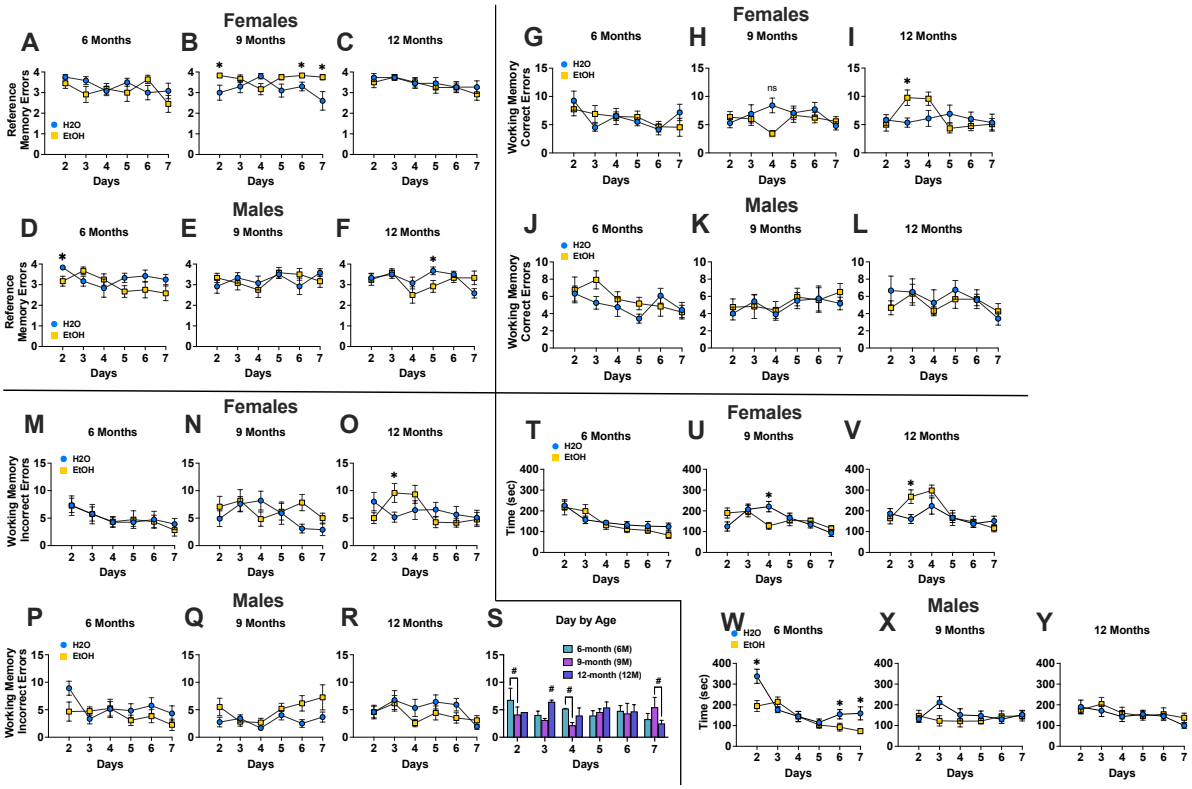


Figure 5.7. Depiction of the results for the observed behavior in the Radial Arm Water Maze for female and male mice. A notable Day by Age x Drinking History interaction for the female mice was deconstructed along the Age factor and revealed (**A**) no significant differences for the 6M and (**C**) 12M females. However, (**B**) a significant Day by Drinking History interaction was observed, indicating that binge-drinking females made more reference memory errors on days 2, 6, and 7 compared to water-drinking females. For the male mice, (**D**) 6M binge-drinking mice made fewer errors on day 2 compared their age-matched water-drinking counterparts. (**E**) No significant group differences were observed at 9M. (**F**) 12M binge-drinking males exhibited fewer errors on day 5 compared to water-drinking counterparts. Results for the female mice indicated a significant Day by Age by Drinking History interaction which was then deconstructed along the Age factor. Concerning working memory errors, (**G**) results for the 6M female mice showed a main effect of day, but no significant interaction. (**H**) For the 9M females, a notable Day by Drinking History trend indicated that binge-drinking females committed fewer working memory correct errors on day 4 relative to their water-drinking counterparts. Lastly, (**I**) for the 12M females, a significant Day by Drinking History interaction revealed that, on day 3, females with a history of binge-drinking committed more errors than their alcohol naïve counterparts. (**J-L**) No significant interactions or main effects were discovered for the male mice. Results for working memory incorrect demonstrated that (**M**) the 6M females showed a noteworthy main effect of Day, but no significant interaction effect. (**N**) Conversely, no significant differences were observed for the 9M females. (**O**) A significant Day by Drinking History interaction

was observed for the 12M females, revealing an alcohol-water differences in number of errors on day 3. (**P-R**) For the male mice, no significant Day by Age by Drinking History interaction was detected, but (**S**) a significant Day by Age interaction was further examined by collapsing along the Age factor sowing that on days 2 and 4, 6M mice committed more errors than the 9M mice. On day 3, 12M mice committed the most errors and on day 7, 9M mice showed more errors than the 12M mice. Considering the time taken to complete the maze, (**T**) no significant group difference were observed for the 6M female mice. (**U**) However, on day 4, 9M water-drinking females take longer to complete the training session. (**V**) For the 12M binge-drinking females exhibited longer completion times than their water-drinking counterparts on day 3. For the male mice, (**W**) 6M binge-drinking mice generally complete the maze quicker on days 2, 6 and 7 of training, while no significant performance variations are observed for (**X**) 9M and (**Y**) 12M males. Figures show means ± SEMs. *p < 0.05, EtOH vs. H20; #p < 0.05, age difference

5.3.8 Immunoblotting

The immunoblotting results below are organized by brain region. For the sake of clarity, only statistically significant outcomes are highlighted in the main text. Full statistical details, including null results, are provided in **Tables 5.1-5.3.**

5.3.8.1 Entorhinal Cortex

A	**Glutamate Related Proteins - Entorhinal Cortex**			
Protein of Interest	**Age (months)**	**Main Effect: Drinking History**	**Interaction Effect: Sex by Drinking History**	**Significant Group Comparisons**
mGlu1	6M	Not Significant	**Significant** $F(1,34) = 7.71$, $p = .009$, $\eta^2 = .185$	Female EtOH = Female H2O ($p = .342$) **Male EtOH > Male H2O ($p = .006$)** **Female EtOH < Male EtOH ($p < .001$)** Female H2O = Male H2O ($p = 1.000$)
	9M	Not Significant $p = .255$, $\eta^2 = .039$	Not Significant $F(1,33) = 3.74$, $p = .062$, $\eta^2 = .102$	None
	12M	Not Significant $p = .960$, $\eta^2 = .000$	Not Significant $F(1,31) = 1.11$, $p = .301$, $\eta^2 = .034$	None
mGlu5 Dimer	6M	Not Significant $p = .737$, $\eta^2 = .003$	Not Significant	None

			$F(1,39) = 0.01$, $p = .927$, $\eta^2 = .000$	
	9M	Not Significant $p = .376$, $\eta^2 = .024$	Not Significant $F(1,33) = 1.04$, $p = .315$, $\eta^2 = .031$	None
	12M	Not Significant $p = .262$, $\eta^2 = .043$	Not Significant $F(1,29 = 0.14$, $p = .716$, $\eta^2 = .005$	None
mGlu5 Monomer	6M	Not Significant $p = .771$, $\eta^2 = .002$	Not Significant $F(1,35) = 1.99$, $p = .167$, $\eta^2 = .054$	None
	9M	**Significant** $F(1,34) = 4.73$, $p = .037$, $\eta^2 = .122$	Not Significant $F(1,34) = 1.02$, $p = .319$, $\eta^2 = .029$	**EtOH < H2O ($p = .037$)**
	12M	**Significant** $F(1,27) = 12.59$, $p = .001$, $\eta^2 = .318$	Not Significant $F(1,27) = 2.04$, $p = .164$, $\eta^2 = .070$	**EtOH > H2O ($p = .001$)**
GluN1	6M	Not Significant $p = .065$, $\eta^2 = .087$	Not Significant $F(1,38) = 0.63$, $p = .434$, $\eta^2 = .016$	None
	9M	Not Significant $p = .967$, $\eta^2 = .000$	Not Significant $F(1,38) = 0.07$, $p = .797$, $\eta^2 = .002$	None
	12M	Not Significant $p = .245$, $\eta^2 = .050$	**Significant** $F(1,27) = 1.91$, $p = .010$, $\eta^2 = .220$	Female EtOH = Female H2O ($p = .317$) **Male EtOH > Male H2O ($p = .005$)** **Female EtOH < Male EtOH ($p = .001$)** Female H2O = Male H2O ($p = 1.000$)
GluN2B	6M	Not Significant $p = .075$, $\eta^2 = .003$	Not Significant $F(1,40) = 0.68$, $p = .416$, $\eta^2 = .017$	None
	9M	**Significant** $F(1,34) = 7.71$, $p = .002$, $\eta^2 = .244$	**Significant** $F(1,34) = 7.71$, $p = .009$, $\eta^2 = .185$	Female EtOH = Female H2O ($p = .681$) **Male EtOH < Male H2O ($p < .001$)** **Female EtOH > Male EtOH ($p < .001$)** Female H2O = Male H2O ($p = 1.000$)
	12M	Not Significant $p = .123$, $\eta^2 = .092$	**Significant** $F(1,25) = 11.24$, $p = .003$, $\eta^2 = .310$	Female EtOH = Female H2O ($p = .270$) **Male EtOH > Male H2O ($p < .001$)** **Female EtOH < Male EtOH ($p < .001$)** Female H2O = Male H2O ($p = 1.000$)
Homer	6M	Not Significant	Not Significant	None

1 b/c		p = .758, η^2 = .003	F(1,37) = 0.16, p = .693, η^2 = .004	
	9M	Not Significant p = .596, η^2 = .009	Not Significant F(1,32) = 0.47, p = .499, η^2 = .014	None
	12M	Not Significant p = .191, η^2 = .053	Not Significant F(1,32) = 0.53, p = .474, η^2 = .016	None
Homer 2 a/b	6M	Not Significant p = .560, η^2 = .010	Not Significant F(1,36) = 0.08, p = .738, η^2 = .002	None
	9M	Not Significant p = .642, η^2 = .007	Not Significant F(1,32) = 1.76, p = .195, η^2 = .052	None
	12M	**Significant** F(1,25) = 4.59, p = .042, η^2 = .155	Not Significant F(1,25) = 1.58, p = .221, η^2 = .059	**EtOH > H2O (p = .042)**
ERK	6M	Not Significant p = .069, η^2 = .080	Not Significant F(1,40) = 0.71, p = .406, η^2 = .017	None
	9M	Not **Significant** p = .600, η^2 = .008	Not Significant F(1,33) = 0.93, p = .342, η^2 = .027	None
	12M	**Significant** F(1,28) = 8.59, p = 0.007, η^2 = .235	**Significant** F(1,28) = 15.52, p <.001, η^2 = .357	**Female EtOH < Female H2O (p <.001)** Male EtOH = Male H2O (p = .400) **Female EtOH < Male EtOH (p <.001)** Female H2O = Male H2O (p = 1.000)
pERK	6M	Not Significant p = .281, η^2 = .040	Not Significant F(1,29) = 0.40, p = .531, η^2 = .014	None
	9M	**Significant** F(1,31) = 5.70, p = .023, η^2 = .155	**Significant** F(1,31) = 7.30, p = .011, η^2 = .191	Female EtOH = Female H2O (p = .827) **Male EtOH > Male H2O (p = .001)** **Female EtOH < Male EtOH (p <.001)** Female H2O = Male H2O (p = 1.000)
	12M	Not Significant p = .717, η^2 = .005	Not Significant F(1,29) = 1.75, p = .196, η^2 = .057	None
B	**Neuropathological Protein Expression – Entorhinal Cortex**			
Protein of Interest	**Age (months)**	**Main Effect: Drinking History**	**Interaction Effect: Sex by Drinking History**	**Significant Group Comparisons**
Tau	6M	Not Significant	Not Significant	None

		p = .288, η^2 = .032	F(1,35) = 1.15, p = .291, η^2 = .032	
	9M	Not Significant p = .550, η^2 = .011	Not Significant F(1,33) = 0.55, p = .463, η^2 = .016	None
	12M	Not Significant p = .053, η^2 = .132	Not Significant F(1,27) = 0.03, p = .877, η^2 = .001	None
pThr(217) Tau	6M	Not Significant p = .840, η^2 = .001	Not Significant F(1,34) = 0.04, p = .838, η^2 = .001	None
	9M	**Significant** F(1,33) = 6.75, p = .014, η^2 = .170	**Significant** F(1,33) = 5.96, p = .020, η^2 = .153	Female EtOH = Female H2O (p = .908) **Male EtOH > Male H2O (p = .002)** **Female EtOH < Male EtOH (p < .001)** Female H2O = Male H2O (p = 1.000)
	12M	**Significant** F(1,28) = 16.51, p <.001, η^2 = .371	Not Significant F(1,28) = 2.26, p = .144, η^2 = .075	**EtOH > H2O (p <.001)**
pSer(396) Tau	6M	Not Significant p = .934, η^2 = .000	Not Significant F(1,37) = 1.03, p = .317, η^2 = .027	None
	9M	**Significant** F(1,33) = 15.17, p <.001, η^2 = .315	Not Significant F(1,33) = 1.99, p = .168, η^2 = .057	**EtOH < H2O (p <.001)**
	12M	Not Significant p = .349, η^2 = .030	Not Significant F(1,23) = 3.43, p = .077, η^2 = .111	None
BACE 56 kDa	6M	**Significant** F(1,32) = 5.27, p = .028, η^2 = .141	Not Significant F(1,32) = 0.82, p = .371, η^2 = .025	**EtOH < H2O (p = .028)**
	9M	Not Significant p = .609, η^2 = .008	Not Significant F(1,32) = 0.174, p = .679, η^2 = .005	None
	12M	Not Significant p = .172, η^2 = .061	Not Significant F(1,26) = 2.32, p = .140, η^2 = .071	None
BACE 70 kDa	6M	**Significant** F(1,36) = 17.63, p <.001, η^2 = .329	Not Significant F(1,36) = 0.12, p = .731, η^2 = .003	**EtOH < H2O (p <.001)**
	9M	Not Significant p = .606, η^2 = .009	Not Significant F(1,31) = 0.52, p = .476, η^2 = .016	None
	12M	**Significant** F(1,28) = 5.53, p = .026, η^2 = .157	Not Significant F(1,28) = 0.79, p = .380, η^2 = .023	**EtOH > H2O (p = .026)**
APP	6M	Not Significant	Not Significant	None

		p = .191, η^2 = .044	F(1,39) = 0.23, p = .638, η^2 = .006	
	9M	Not Significant p = .476, η^2 = .014	Not Significant F(1,36) = 0.00, p = .958, η^2 = .000	None
	12M	**Significant** F(1,32) = 15.82, p <.001, η^2 = .331	Not Significant F(1,32) = 0.47, p = .500, η^2 = .014	**EtOH > H2O (p <.001)**
Aβ	6M	Not Significant p = .081, η^2 = .089	Not Significant F(1,33) = 2.94, p = .096, η^2 = .082	None
	9M	**Significant** F(1,37) = 10.09, p = .003, η^2 = .214	Not Significant F(1,37) = 0.04, p = .836, η^2 = .001	**EtOH < H2O (p = .003)**
	12M	Not Significant p = .328, η^2 = .029	Not Significant F(1,33) = 3.70, p = .063, η^2 = .101	None

Table 5.1. Protein Expression in the Entorhinal Cortex. Summary of the quantitative analysis of proteins implicated in (A) glutamate function (B) and neuropathological processes.
Significant results are bolded.

Group1 mGluRs. A significant interaction between Sex and Drinking History was detected for mGlu1 expression only in 6M mice [$F(1,34) = 7.71$, $p = 0.009$, $\eta^2 = 0.185$; **Figure 5.8A**]. LSD tests for simple main effects showed that 6M binge-drinking male mice exhibited a significantly higher mGlu1 expression versus both their water-drinking counterparts ($p = 0.006$) and the female binge-drinking mice ($p < 0.001$). In contrast, no significant interactions, or main effects for mGlu1 were detected in the 9M or 12M mice (see **Table 5.1A**; **Figure 5.8A' & A"**). No significant Sex x Drinking History interactions were detected for either the mGlu5 monomer (**Figure 5.8B-B")** or dimer (**Figure 5.8C-C")** in mice of any age (see **Table 5.1A**). Although no water-alcohol difference in mGlu5 expression was apparent in 6M mice ($p = 0.771$, $\eta^2 = 0.002$), a significant main Drinking History effect was detected for mGlu5 monomer expression in both 9M [$F(1,34) = 4.73$, $p = 0.037$, $\eta^2 = 0.122$;

H2O > EtOH; **Figure 5.8C'**] and 12M mice [$F(1,27) = 12.59$, $p = 0.001$, $\eta^2 = 0.318$; EtOH > H2O; **Figure 5.8C"**].

NMDA subunits. A Sex x Drinking History ANOVA indicated no significant interaction or main effects for the expression of the NMDA receptor subunits GluN1 (see **Table 5.1A**; **Figure 5.8D)** and GluN2B (see **Table 5.1A**; **Figure 5.8E)** in 6M mice. However, a significant interaction for GluN2B was detected in 9M mice [$F(1,32) = 5.47$, $p = 0.009$, $\eta^2 = 0.185$; **Figure 5.8E'**] and LSD tests for simple main effects indicated lower GluN2B expression in binge-drinking males versus both their water controls ($p < 0.001$), and binge-drinking females ($p < 0.001$). Additionally, significant interactions for both GluN1 and GluN2B subunits were detected in 12M mice [GluN1: $F(1,27) = 5.91$, $p = 0.010$, $\eta^2 = 0.220$; GluN2B: $F(1,25) = 11.24$, $p = 0.003$, $\eta^2 = 0.310$]. Analysis of simple main effects revealed that 12M binge-drinking mice exhibited higher levels of both GluN1 and GluN2B than both male water-drinking controls (GluN1: $p = 0.006$; GluN2B: $p < 0.001$) and female alcohol-drinking mice (GluN1: $p < 0.001$; GluN2B: $p < 0.001$), as shown in **Figures 5.8D" and 5.8E"**, respectively.

Homer proteins. Two-way ANOVAs failed to detect any significant interactions for either Homer 1b/c or Homer 2a/b expression at any of the ages tested (see **Table 5.1A**), although a significant main effect of Drinking History was detected for Homer2 levels in the 12M mice [$F(1,25) = 4.59$, $p = 0.042$, $\eta^2 = 0.155$; EtOH > H2O; **Figure 5.8G").**

ERK. Sex x Drinking History ANOVAs indicated no main effects or interactions for ERK in 6M, 9M, or 12M mice, or for p(Tyr204)-ERK in 6M mice (see **Table 5.1B**). However, a significant interaction was observed for p(Tyr204)-ERK in 9M mice [$F(1,31) = 7.30$, $p = 0.011$, $\eta^2 = 0.191$; **Figure 5.8I'**] that reflect higher phospho-ERK levels in binge-drinking males, compared to both the male water-drinking mice ($p = 0.001$) and female binge-drinking mice ($p < 0.001$). While no significant interactions were detected for p(Tyr204)-ERK in 12M animals, an interaction was detected for ERK expression in this age group [$F(1,28) = 15.52$, $p < 0.001$, $\eta^2 = 0.357$; H2O > EtOH; **Figure 5.8H"**].

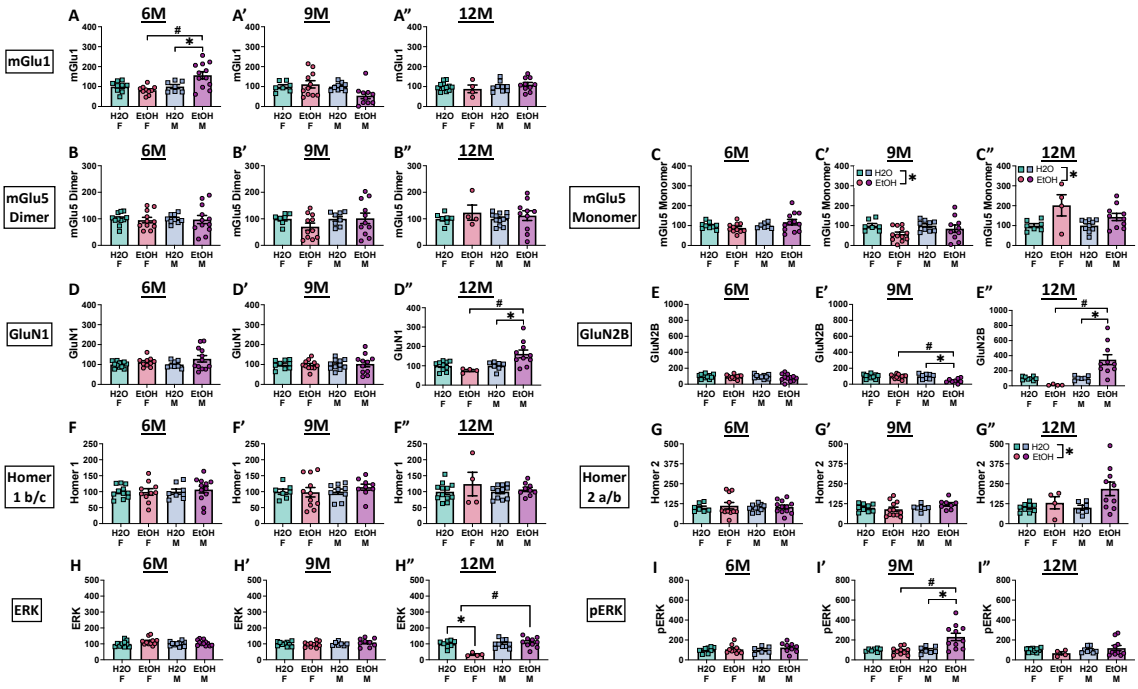


Figure 5.8. Summary of the effects of Sex and Drinking History on the expression of glutamate-related proteins in the entorhinal cortex. Group 1 mGlu receptors: (**A-A"**) mGlu1, (**B-B"**) mGlu5 (dimer), and (**C-C"**) mGlu5 (monomer), the NMDA receptor subunits: (**D-D"**) GluN1, (**E-E"**) GluN2B, (**F-F"**) Homer 1b/c and (**G-G"**) Homer 2a/b, (**H-H"**) ERK and (**I-I"**) pERK. Data represent means ± SEMs, with specific significant interactions highlighted. Figures show means ± SEMs. *p < 0.05, EtOH vs. H20; #p < 0.05, age difference

Tau proteins. Sex x Drinking History ANOVAs failed to detect any main effects or interactions regarding Tau protein expression in mice of any age (see **Table 5.1B**; **Figure 5.9A – A"**). A Sex x Drinking History ANOVA failed to indicate any group differences in the expression of p(Thr217)-Tau (see **Table 5.1B**; **Figure 5.9B**) in 6M mice. However, a significant Sex x Drinking History interaction was detected in 9M mice [$F(1,33) = 5.96$, $p = 0.020$, $\eta^2 = 0.153$; **Figure 5.9B'**] and simple main effects analysis indicated revealed elevated p(Thr217)-Tau levels in binge-drinking males, relative to both their male water-drinking controls ($p = 0.002$) and female alcohol-drinking counterparts ($p < 0.001$). A significant Drinking History effect was also detected in 12M mice [$F(1,28) = 16.51$, $p < 0.001$, $\eta^2 = 0.371$; EtOH > H2O; **Figure 5.9B"**]. Similar to p(Thr217)-Tau, analysis failed to detect any significant main effects of interactions in the expression of p(Ser396)-Tau (see **Table 5.1B**, **Figure 5.9C**) in 6M mice. In contrast, a significant main effect of Drinking History was found in 9M [$F(1,33) = 15.17$, $p < 0.001$, $\eta^2 = 0.315$; H2O > EtOH; **Figure 5.9C'**]. No significant changes in p(Ser396)-Tau levels were detected in the 12M mice (see **Table 5.1B**; **Figure 5.9C"**).

BACE isoforms. Sex x Drinking ANOVAs failed to detect any interactions for either BACE isoform at any of the ages examined (**Table 5.1B**). However, significant main Drinking History effects were observed in 6M mice for both BACE 56kDa [$F(1,32) = 5.27$, $p = 0.028$, $\eta^2 = 0.141$; H2O > EtOH; **Figure 5.9D**] and BACE 70kDa [$F(1,36) = 17.63$, $p < 0.001$, $\eta^2 = 0.329$; H2O > EtOH; **Figure 5.9E**]. In contrast, no significant main effects for either BACE isoform were detected in 9M mice (see **Table 5.1B**), while a significant Drinking History

effect was observed for BACE 70kDa in 12M animals [$F(1,28) = 5.53$, $p = 0.026$, $\eta^2 = 0.157$; H2O < EtOH; **Figure 5.9E"**].

APP and AB. Sex x Drinking History ANOVAs indicated no significant interactions for APP expression in any age (see **Table 5.1B**). However, a significant main Drinking History effect was observed in 12M mice [$F(1,34) = 15.82$, $p < 0.001$, eta = .331; H2O < EtOH; **Figure 5.9F"**]. Akin to our results for APP, Sex x Drinking History ANOVAs failed to indicate any significant interaction for amyloid-beta in any age group (see **Table 5.1B**). However, we did detect a significant main Drinking History effect of Drinking History in 9M mice [$F(1,37) = 10.09$, $p = 0.003$, $\eta^2 = 0.241$; H2O > EtOH; **Figure 5.9G"**).

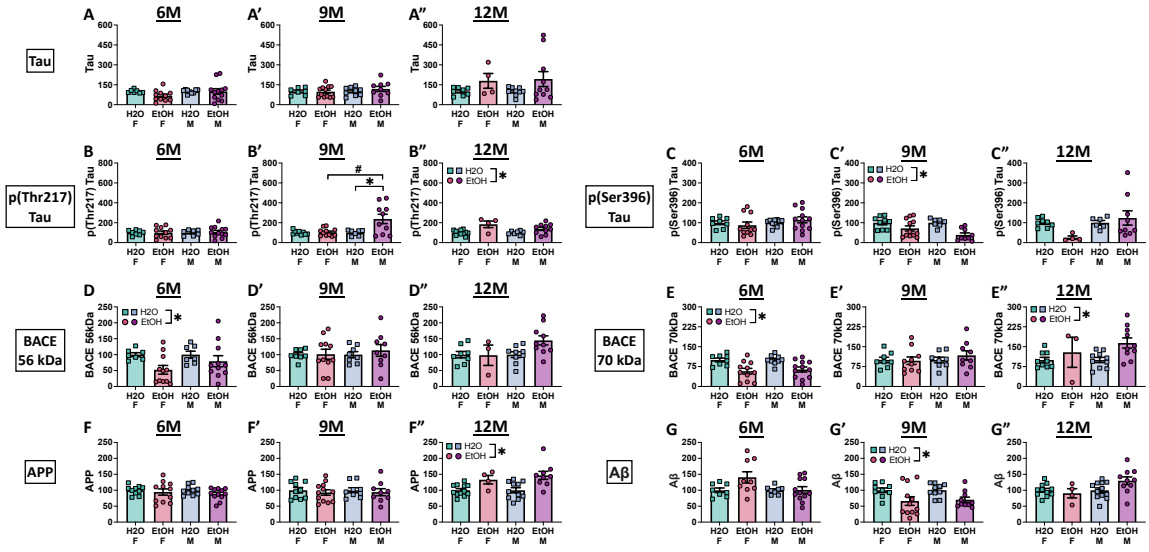


Figure 5.9. Summary of the effects of Sex and Drinking History on the expression of indices of neuropathology in the entorhinal cortex. Tau proteins: (**A-A"**) Tau, (**B-B"**) p(Thr217)-Tau, and (**C-C"**) p(Ser396)-Tau, the BACE isoforms: (**D-D"**) BACE 56kDa and (**E-E"**) BACE 70kDa, (**F-F"**) APP (**G-G"**). Data represent means ± SEMs, with specific significant interactions highlighted. Figures show means ± SEMs. *p < 0.05, EtOH vs. H20; #p < 0.05, age difference

5.3.8.2 Prefrontal Cortex

A	Glutamate Related Proteins - Prefrontal Cortex			
Protein of Interest	**Age (months)**	**Main Effect: Drinking History**	**Interaction Effect: Sex by Drinking History**	**Significant Group Comparisons**
mGlu1	6M	**Significant** $F(1,37) = 4.59$, $p = .039$, $\eta^2 = .110$	Not Significant $F(1,37) = 1.06$, $p = .311$, $\eta^2 = .028$	**EtOH > H2O ($p = .039$)**
	9M	Not Significant $p = .318$, $\eta^2 = .024$	Not Significant $F(1,42) = 0.01$, $p = .915$, $\eta^2 = .000$	None
	12M	Not Significant $p = .922$, $\eta^2 = .000$	Not Significant $F(1,32) = 3.84$, $p = .059$, $\eta^2 = .107$	None
mGlu5 Dimer	6M	Not Significant $p = .155$, $\eta^2 = .057$	Not Significant $F(1,35) = 0.38$, $p = .543$, $\eta^2 = .011$	None
	9M	Not Significant $p = .330$, $\eta^2 = .025$	Not Significant $F(1,38) = 0.21$, $p = .650$, $\eta^2 = .005$	None
	12M	Not Significant $p = .806$, $\eta^2 = .002$	Not Significant $F(1,28) = 0.41$, $p = .527$, $\eta^2 = .014$	None
mGlu5 Monomer	6M	**Significant** $F(1,33) = 6.41$, $p = .016$, $\eta^2 = .163$	Not Significant $F(1,33) = 0.53$, $p = .470$, $\eta^2 = .016$	**EtOH > H2O ($p = .016$)**
	9M	Not Significant $p = .159$, $\eta^2 = .050$	**Significant** $F(1,39) = 5.40$, $p = .025$, $\eta^2 = .122$	Female EtOH = Female H2O ($p = .540$) **Male EtOH < Male H2O ($p = .010$)** **Female EtOH > Male EtOH ($p = .001$)** Female H2O = Male H2O ($p = 1.000$)
	12M	Not Significant $p = .535$, $\eta^2 = .013$	Not Significant $F(1,30) = 0.03$, $p = .957$, $\eta^2 = .000$	None
GluN1	6M	Not Significant $p = .062$, $\eta^2 = .089$	Not Significant $F(1,38) = 3.71$, $p = .062$, $\eta^2 = .089$	None
	9M	**Significant** $F(1,40) = 5.02$, $p = .031$, $\eta^2 = .112$	Not Significant $F(1,40) = 3.79$, $p = .059$, $\eta^2 = .086$	**EtOH < H2O ($p = .031$)**
	12M	Not Significant $p = .282$, $\eta^2 = .032$	Not Significant $F(1,36) = 1.23$, $p = .276$, $\eta^2 = .033$	None
GluN2B	6M	**Significant**	Not Significant	**EtOH > H2O ($p = .004$)**

		$F(1,34) = 9.36$, $p = .004$, $\eta^2 = .216$	$F(1,34) = 1.76$, $p = .194$, $\eta^2 = .049$	
	9M	Not Significant $P = .199$, $\eta^2 = .049$	**Significant** $F(1,33) = 13.36$, $p < .001$, $\eta^2 = .288$	**Female EtOH < Female H2O ($p = .002$)** Male EtOH = Male H2O ($p = .086$) **Female EtOH < Male EtOH ($p < .001$)** Female H2O = Male H2O ($p = 1.000$)
	12M	Not Significant $p = .535$, $\eta^2 = .013$	Not Significant $F(1,29) = .355$, $p = .556$, $\eta^2 = .012$	None
Homer 1 b/c	6M	**Significant** $F(1,35) = 19.09$, $p < .001$, $\eta^2 = .353$	Not Significant $F(1,35) = 1.75$, $p = .195$, $\eta^2 = .048$	**EtOH > H2O ($p < .001$)**
	9M	Not Significant $p = .813$, $\eta^2 = .001$	Not Significant $F(1,38) = 3.37$, $p = .074$, $\eta^2 = .081$	None
	12M	Not Significant $p = .409$, $\eta^2 = .022$	Not Significant $F(1,31) = .47$, $p = .500$, $\eta^2 = .015$	None
Homer 2 a/b	6M	**Significant** $F(1,38) = 16.93$, $p < .001$, $\eta^2 = .308$	Not Significant $F(1,38) = 0.78$, $p = .383$, $\eta^2 = .020$	**EtOH > H2O ($p < .001$)**
	9M	Not Significant $p = .214$, $\eta^2 = .038$	Not Significant $F(1,40) = 1.37$, $p = .249$, $\eta^2 = .033$	None
	12M	Not Significant $p = .115$, $\eta^2 = .076$	Not Significant $F(1,32) = .000$, $p = .986$, $\eta^2 = .000$	None
ERK	6M	**Significant** $F(1,41) = 9.76$, $p = .003$, $\eta^2 = .192$	Not Significant $F(1,41) = 0.63$, $p = .433$, $\eta^2 = .015$	**EtOH > H2O ($p = .003$)**
	9M	Not Significant $p = .169$, $\eta^2 = .047$	Not Significant $F(1,40) = 0.73$, $p = .398$, $\eta^2 = .018$	None
	12M	Not Significant $p = .822$, $\eta^2 = .002$	Not Significant $F(1,33) = .106$, $p = .746$, $\eta^2 = .003$	None
pERK	6M	**Significant** $F(1,35) = 44.94$, $p < .001$, $\eta^2 = .562$	Not Significant $F(1,35) = 2.73$, $p = .107$, $\eta^2 = .072$	**EtOH > H2O ($p < .001$)**
	9M	Not Significant $p = .640$, $\eta^2 = .005$	Not Significant $F(1,41) = 2.52$, $p = .120$, $\eta^2 = .058$	None
	12M	Not Significant $p = .574$, $\eta^2 = .011$	**Significant** $F(1,28) = 4.57$, $p = .042$, $\eta^2 = .140$	Female EtOH = Female H2O ($p = .092$) Male EtOH < Male H2O ($p = .225$)

				Female EtOH > Male EtOH (*p* = .007) Female H2O = Male H2O (*p* = 1.000)
B	**Neuropathological Protein Expression – Prefrontal Cortex**			
Protein of Interest	**Age (months)**	**Main Effect: Drinking History**	**Interaction Effect: Sex by Drinking History**	**Significant Group Comparisons**
Tau	6M	Not Significant $p = .841$, $\eta^2 = .001$	Not Significant $F(1,34) = 0.30$, $p = .590$, $\eta^2 = .009$	None
	9M	Not Significant $p = .101$, $\eta^2 = .073$	Not Significant $F(1,36) = 0.23$, $p = .633$, $\eta^2 = .006$	None
	12M	Not Significant $p = .161$, $\eta^2 = .062$	Not Significant $F(1,31) = .07$, $p = .793$, $\eta^2 = .002$	None
pThr(217) Tau	6M	**Significant** $F(1,34) = 4.86$, $p = .034$, $\eta^2 = .125$	Not Significant $F(1,34) = 0.31$, $p = .580$, $\eta^2 = .009$	**EtOH > H2O (*p* = .034)**
	9M	Not Significant $p = .570$, $\eta^2 = .009$	Not Significant $F(1,36) = 0.31$, $p = .580$, $\eta^2 = .009$	None
	12M	Not Significant $p = .118$, $\eta^2 = .072$	Not Significant $F(1,33) = .10$, $p = .758$, $\eta^2 = .003$	None
pSer(396) Tau	6M	Not Significant $p = .079$, $\eta^2 = .090$	Not Significant $F(1,33) = 1.84$, $p = .184$, $\eta^2 = .053$	None
	9M	Not Significant $p = .066$, $\eta^2 = .086$	Not Significant $F(1,38) = 0.05$, $p = .817$, $\eta^2 = .001$	None
	12M	**Significant** $F(1,28) = 10.01$, $p = .004$, $\eta^2 = .263$	Not Significant $F(1,28) = .93$, $p = .342$, $\eta^2 = .032$	**EtOH < H2O (*p* = .004)**
BACE 56 kDa	6M	Not Significant $p = .243$, $\eta^2 = .041$	Not Significant $F(1,33) = 0.11$, $p = .743$, $\eta^2 = .003$	None
	9M	Not Significant $p = .118$, $\eta^2 = .067$	Not Significant $F(1,36) = 0.25$, $p = .621$, $\eta^2 = .007$	None
	12M	Not Significant $p = .887$, $\eta^2 = .001$	Not Significant $F(1,34) = 1.38$, $p = .249$, $\eta^2 = .039$	None
BACE 70 kDa	6M	Not Significant $p = .179$, $\eta^2 = .052$	Not Significant $F(1,34) = 0.35$, $p = .558$, $\eta^2 = .010$	None
	9M	Not Significant $p = .910$, $\eta^2 = .000$	Not Significant $F(1,42) = 0.10$, $p = .754$, $\eta^2 = .002$	None

	12M	Not Significant $p = .708$, $\eta^2 = .004$	Not Significant $F(1,37) = .79$, $p = .379$, $\eta^2 = .021$	None
APP	6M	Not Significant $p = .330$, $\eta^2 = .024$	Not Significant $F(1,39) = 0.03$, $p = .854$, $\eta^2 = .001$	None
	9M	Not Significant $p = .382$, $\eta^2 = .021$	Not Significant $F(1,37) = 0.01$, $p = .922$, $\eta^2 = .000$	None
	12M	**Significant** $F(1,33) = 5.36$, $p = .027$, $\eta^2 = .140$	Not Significant $F(1,33) = 1.20$, $p = .282$, $\eta^2 = .035$	**EtOH > H2O ($p = .027$)**
Aβ	6M	Not Significant $p = .552$, $\eta^2 = .010$	Not Significant $F(1,35) = 0.25$, $p = .619$, $\eta^2 = .007$	None
	9M	Not Significant $p = .903$, $\eta^2 = .000$	Not Significant $F(1,34) = 1.05$, $p = .313$, $\eta^2 = .030$	None
	12M	Not Significant $p = .945$, $\eta^2 = .000$	Not Significant $F(1,36) = .19$, $p = .666$, $\eta^2 = .005$	None

Table 5.2. Protein Expression in the Prefrontal Cortex. Summary of the quantitative analysis of proteins implicated in (A) glutamate function (B) and neuropathological processes. Significant results are bolded.

Group1 mGluRs. No significant Sex x Drinking History interaction was detected for PFC mGlu1 expression in mice of any age (see B). Nevertheless, a significant main effect of Drinking History was detected for 6M mice [$F(1,37) = 4.59$, $p = .039$, $\eta^2 = .110$; **Figure 5.10A**] that reflected elevated mGlu1 expression in binge-drinking mice of both sexes, compared to their water-drinking counterparts. No significant Sex x Drinking History interaction was observed for PFC mGlu5 dimer expression in 6M, 9M, or 12M mice (see **Table 5.2A**; **Figures 5.10B-B"**). However, a significant main Drinking History effect was detected for 6M mice [$F(1,33) = 6.41$, $p = 0.016$, $\eta^2 = 0.163$; EtOH > H20; **Figure 5.10C**). In contrast, a significant Sex x Drinking History interaction was observed for mGlu5 monomer levels for the 9M mice [$F(1,39) = 5.40$, $p = 0.025$, $\eta^2 = 0.122$; **Figure 5.10C'**] and LSD tests

for simple main effects detected elevated mGlu5 monomer levels in male water- versus male binge-drinking mice ($p = 0.010$), in addition to higher mGlu5 monomer expression in female versus male binge-drinking mice ($p = 0.001$). Additionally, No significant main effects were detected for the 12M mice (see **Table 5.2A**; **Figure 5.10C"**).

NMDA subunits. A Sex x Drinking History ANOVA indicated no significant main effects or interactions for PFC GluN1 expression in 6M or 12M mice (see **Table 5.2A**). However, a significant main Drinking History effect on GluN1 was detected for the 9M mice [$F(1,40) = 5.02$, $p = 0.031$, $\eta^2 = 0.112$; H2O > EtOH; **Figure 5.10D'**]. A significant main Drinking History effect was identified for GluN2b expression in 6M mice [$F(1,34) = 9.36$, $p = 0.004$, $\eta^2 = 0.216$; EtOH > H20; **Figure 5.10E]**, while a significant interaction was detected in 9M mice [$F(1,33) = 13.36$, $p < 0.001$, $\eta^2 = 0.288$; **Figure 5.10E'**], that reflected lower GluN2B expression in female binge-drinking mice, relative to both their female water-drinking counterparts ($p = 0.002$) and the male binge-drinking mice ($p < 0.001$). No significant main effects or interactions were observed for GluN2b expression in the 12M mice (see **Table 5.2A**, **Figure 5.10E"**).

Homer proteins. A significant main Drinking History effect was observed for both Homer 1b/c [$F(1,35) = 19.09$, $p < 0.001$, $\eta^2 = 0.353$; EtOH > H2O; **Figure 5.10F**] and Homer 2a/b [$F(1,38) = 16.93$, $p < 0.001$, $\eta^2 = 0.308$; EtOH > H2O; **Figure 5.10G**] expression within the PFC of 6M mice. In contrast, Sex x Drinking History ANOVAs failed to detect any significant main effects or interactions for either Homer protein in 9M or 12M mice (see **Table 5.2A**).

ERK. A significant main Drinking History effect was observed for ERK expression in the PFC [$F(1,41) = 9.76$, $p = 0.003$, $\eta^2 = 0.192$; EtOH > H2O; **Figure 5.10H**]. In contrast, Sex x Drinking History ANOVAs failed to detect any main effects or interactions for ERK in 9M and 12M or for p(Tyr204)-ERK in 9M mice (see **Table 5.2B**; **Figures 5.10H' & H"**). Similarly, a significant main effect of Drinking History was observed for PFC p(Tyr204)-ERK expression in 6M mice [$F(1,35) = 44.94$, $p < 0.001$, $\eta^2 = 0.562$; EtOH > H2O; **Figure 5.10I**]. Moreover, a significant Sex x Drinking History interaction for p(Tyr204)-ERK expression was observed in 12M mice [$F(1,28) = 4.57$, $p = 0.042$, $\eta^2 = 0.140$; **Figure 5.10I"**], which reflected higher phospho-ERK expression for the female binge-drinking mice when compared to the their male binge-drinking counterparts ($p = 0.007$). No significant main effects or interactions were observed for p(Tyr204)-ERK expression in the 12M mice (see **Table 5.2B**, **Figure 5.10I"**).

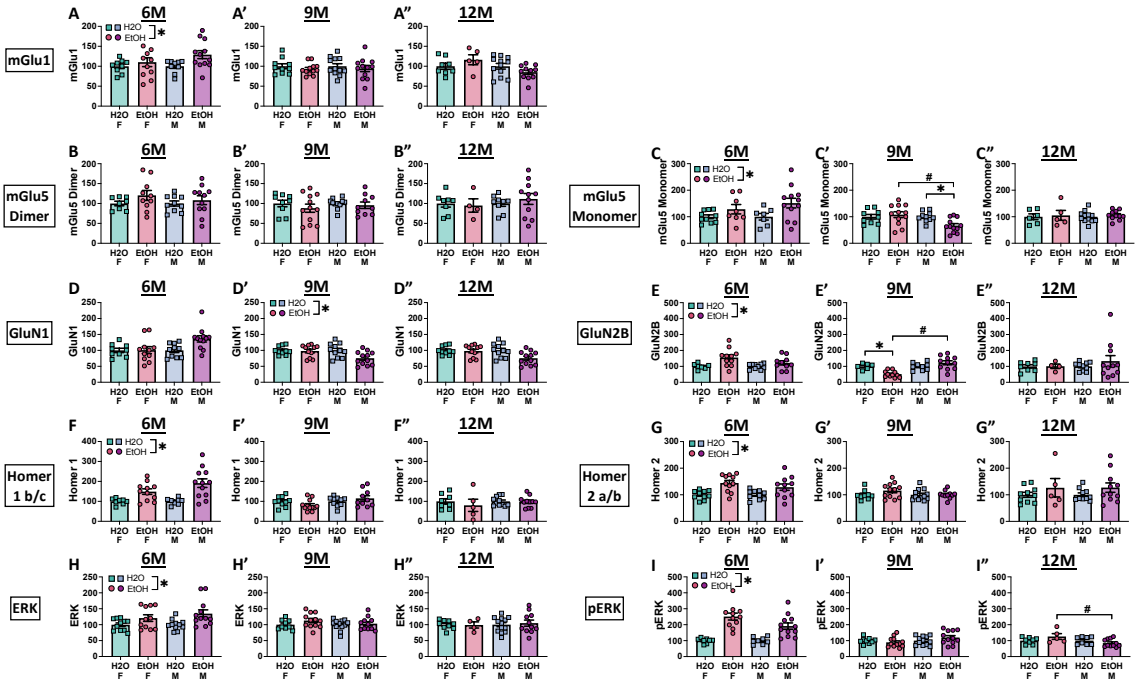


Figure 5.10. Summary of the effects of Sex and Drinking History on the expression of glutamate-related proteins in the prefrontal cortex. Group 1 mGlu receptors: (**A-A"**) mGlu1, (**B-B"**) mGlu5 (dimer), and (**C-C"**) mGlu5 (monomer), the NMDA receptor subunits: (**D-D"**) GluN1, (**E-E"**) GluN2B, (**F-F"**) Homer 1b/c and (**G-G"**) Homer 2a/b, (**H-H"**) ERK and (**I-I"**) pERK. Data represent means ± SEMs, with specific significant interactions highlighted. Figures show means ± SEMs. *p < 0.05, EtOH vs. H20; #p < 0.05, age difference

Tau protein. Sex x Drinking History ANOVAs failed to detect any significant main effects or interactions for Tau protein expression in the PFC of 6M, 9M, or 12M mice (see **Table 5.2B; Figure 5.11A-A"**). A significant main Drinking History effect was detected for PFC expression of p(Thr217)-Tau in 6M mice [$F(1,34) = 4.86$, $p = 0.034$, $\eta^2 = 0.125$; EtOH > H2O; **Figure 5.11B**]. However, Sex x Drinking History ANOVAs failed to indicate any significant group differences in the expression of phosphorylated Tau at threonine 217 [(p(Thr217) Tau] in 9M and 12M mice (see **Table 5.2B**). Sex x Drinking History ANOVAs failed to reveal significant differences in the expression of phosphorylated Tau at serine 396 [p(Ser396) Tau] in 6M and 9M mice (see **Table 5.2B**). Nevertheless, for the 12M cohort, the

2-way ANOVA detected a significant main effect to Drinking History, with binge-drinking mice exhibiting lower levels of p(Ser396) Tau in comparison to their water-drinking counterparts [$F(1,28) = 10.01$, $p = 0.004$, $\eta^2 = 0.263$; **Figure 5.11C"**].

BACE isoforms. Two-way ANOVAs failed to detect any significant main effects or interactions for either BACE isoform in 6M, 9M, or 12M mice (all p's > 0.179; see **Table 5.2B**)

APP and AB. A Sex x Drinking History ANOVA failed to indicate any significant group differences for APP expression in 6M or 9M mice (see **Table 5.2B**). In contrast, a significant Drinking History effect was observed in the PFC of 12M mice [$F(1,33) = 5.36$, $p = 0.027$, $\eta^2 = 0.140$; EtOH > H2O; **Figure 5.11F"**]. No significant Sex x Drinking History main effects or interactions were found for AB in 6M, 9M, or 12M mice (all p's > 0.313; see **Table 5.2B**).

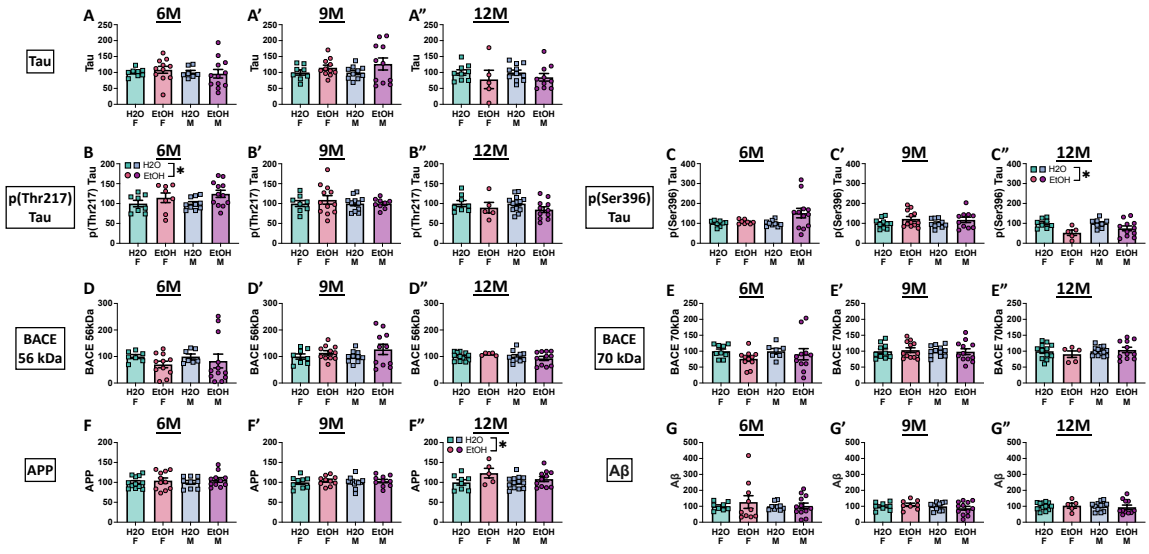


Figure 5.11. Summary of the effects of Sex and Drinking History on the expression of indices of neuropathology in the prefrontal cortex. Tau proteins: (**A-A"**) Tau, (**B-B"**) p(Thr217)-Tau, and (**C-C"**) p(Ser396)-Tau, the BACE isoforms: (**D-D"**) BACE 56kDa and (**E-E"**) BACE 70kDa, (**F-F"**) APP (**G-G"**). Data represent means ± SEMs, with specific significant interactions highlighted. Figures show means ± SEMs. *p < 0.05, EtOH vs. H20; #p < 0.05, age difference

5.3.8.3 Amygdala

A	**Glutamate Related Proteins - Amygdala**			
Protein of Interest	**Age (months)**	**Main Effect: Drinking History**	**Interaction Effect: Sex by Drinking History**	**Significant Group Comparisons**
mGlu1	6M	**Significant** $F(1,30) = 8.49$, $p = .007$, $\eta^2 = .221$	**Significant** $F(1,30) = 4.50$, $p = .042$, $\eta^2 = .130$	**Female EtOH > Female H2O ($p = .001$)** Male EtOH = Male H2O ($p = .580$) **Female EtOH > Male EtOH ($p = .002$)** Female H2O = Male H2O ($p = 1.000$)
	9M	Not Significant $p = .618$, $\eta^2 = .007$	Not Significant $F(1,34) = 0.81$, $p = .375$, $\eta^2 = .023$	None
	12M	**Significant** $F(1,33) = 7.93$, $p = .008$, $\eta^2 = .194$	Not Significant $F(1,33) = .04$, $p = .848$, $\eta^2 = .001$	**EtOH > H2O ($p = .008$)**
mGlu5 Dimer	6M	Not Significant $p = .360$, $\eta^2 = .024$	**Significant** $F(1,35) = 5.94$, $p = .020$, $\eta^2 = .145$	**Female EtOH > Female H2O ($p = .029$)** Male EtOH = Male H2O ($p = .271$) **Female EtOH > Male EtOH ($p < .001$)** Female H2O = Male H2O ($p = 1.000$)
	9M	**Significant** $F(1,34) = 4.38$, $p = .044$, $\eta^2 = .114$	Not Significant $F(1,34) = 0.75$, $p = .394$, $\eta^2 = .021$	**EtOH < H2O ($p = .044$)**
	12M	**Significant** $F(1,33) = 9.59$, $p = .004$, $\eta^2 = .225$	**Significant** $F(1,33) = 25.93$, $p < .001$, $\eta^2 = .440$	**Female EtOH > Female H2O ($p < .001$)** Male EtOH = Male H2O ($p = .108$) **Female EtOH > Male EtOH ($p < .001$)** Female H2O = Male H2O ($p = 1.000$)
mGlu5 Monomer	6M	Not Significant $p = .378$, $\eta^2 = .022$	Not Significant	None

			$F(1,35) = 0.23$, $p = .637$, $\eta^2 = .006$	
	9M	Not Significant $p = .932$, $\eta^2 = .000$	Not Significant $F(1,33) = 1.85$, $p = .183$, $\eta^2 = .053$	None
	12M	Not Significant $p = .932$, $\eta^2 = .000$	Not Significant $F(1,33) = 1.85$, $p = .183$, $\eta^2 = .053$	None
GluN1	6M	Not Significant $p = .052$, $\eta^2 = .104$	Not Significant $F(1,35) = 0.35$, $p = .561$, $\eta^2 = .010$	None
	9M	Not Significant $p = .139$, $\eta^2 = .063$	Not Significant $F(1,34) = 0.01$, $p = .759$, $\eta^2 = .003$	None
	12M	Not Significant $p = .585$, $\eta^2 = .008$	Not Significant $F(1,37) = 3.09$, $p = .087$, $\eta^2 = .077$	None
GluN2B	6M	Not Significant $p = .362$, $\eta^2 = .025$	Not Significant $F(1,33) = 0.15$, $p = .902$, $\eta^2 = .000$	None
	9M	**Significant** $F(1,30) = 14.53$, $p < .001$, $\eta^2 = .326$	Not Significant $F(1,30) = 0.57$, $p = .456$, $\eta^2 = .019$	**EtOH < H2O ($p < .001$)**
	12M	Not Significant $p = .079$, $\eta^2 = .114$	Not Significant $F(1,26) = 2.10$, $p = .159$, $\eta^2 = .075$	None
Homer 1 b/c	6M	Not Significant $p = .362$, $\eta^2 = .023$	Not Significant $F(1,36) = 0.26$, $p = .616$, $\eta^2 = .007$	None
	9M	Not Significant $p = .128$, $\eta^2 = .069$	Not Significant $F(1,33) = 4.01$, $p = .053$, $\eta^2 = .108$	None
	12M	**Significant** $F(1,34) = 15.55$, $p < .001$, $\eta^2 = .314$	Not Significant $F(1,34) = 0.53$, $p = .473$, $\eta^2 = .015$	**EtOH > H2O ($p < .001$)**
Homer 2 a/b	6M	Not Significant $p = .305$, $\eta^2 = .029$	Not Significant $F(1,36) = 0.25$, $p = .619$, $\eta^2 = .007$	None
	9M	Not Significant $p = .652$, $\eta^2 = .006$	Not Significant $F(1,32) = 3.32$, $p = .078$, $\eta^2 = .094$	None
	12M	Not Significant $p = .658$, $\eta^2 = .007$	Not Significant $F(1,29) = .34$, $p = .564$, $\eta^2 = .012$	None
ERK	6M	Not Significant $p = .830$, $\eta^2 = .001$	Not Significant $F(1,39) = 0.15$, $p = .699$, $\eta^2 = .004$	None
	9M	Not Significant $p = .348$, $\eta^2 = .028$	Not Significant $F(1,32) = 0.00$, $p = .978$, $\eta^2 = .000$	None

	12M	**Significant** $F(1,28) = 12.66$, $p = .001$, $\eta^2 = .311$	Not Significant $F(1,28) = 1.02$, $p = .322$, $\eta^2 = .035$	**EtOH < H2O ($p = .001$)**
pERK	6M	**Significant** $F(1,38) = 14.39$, $p = .001$, $\eta^2 = .275$	Not Significant $F(1,38) = 0.51$, $p = .479$, $\eta^2 = .013$	**EtOH > H2O ($p = .001$)**
	9M	Not Significant $p = .160$, $\eta^2 = .061$	Not Significant $F(1,32) = 1.76$, $p = .194$, $\eta^2 = .052$	None
	12M	**Significant** $F(1,28) = 9.84$, $p = .004$, $\eta^2 = .260$	Not Significant $F(1,28) = 1.20$, $p = .282$, $\eta^2 = .041$	**EtOH < H2O ($p = .004$)**
B	**Neuropathological Protein Expression – Amygdala**			
Protein of Interest	**Age (months)**	**Main Effect: Drinking History**	**Interaction Effect: Sex by Drinking History**	**Significant Group Comparisons**
Tau	6M	**Significant** $F(1,36) = 8.35$, $p = .007$, $\eta^2 = .188$	Not Significant $F(1,36) = 0.39$, $p = .537$, $\eta^2 = .011$	**EtOH < H2O ($p = .007$)**
	9M	Not Significant $p = .646$, $\eta^2 = .007$	Not Significant $F(1,29) = 0.76$, $p = .391$, $\eta^2 = .025$	None
	12M	**Significant** $F(1,27) = 9.30$, $p = .005$, $\eta^2 = .256$	Not Significant $F(1,27) = 3.09$, $p = .090$, $\eta^2 = .103$	**EtOH > H2O ($p = .005$)**
pThr(217) Tau	6M	Not Significant $p = .890$, $\eta^2 = .000$	Not Significant $F(1,39) = 1.87$, $p = .180$, $\eta^2 = .046$	None
	9M	Not Significant $p = .353$, $\eta^2 = .028$	Not Significant $F(1,31) = 2.33$, $p = .137$, $\eta^2 = .070$	None
	12M	Not Significant $p = .124$, $\eta^2 = .070$	Not Significant $F(1,33) = 4.09$, $p = .051$, $\eta^2 = .110$	None
pSer(396) Tau	6M	Not Significant $p = .301$, $\eta^2 = .032$	Not Significant $F(1,33) = 0.07$, $p = .788$, $\eta^2 = .002$	None
	9M	**Significant** $F(1,32) = 11.53$, $p = .002$, $\eta^2 = .265$	Not Significant $F(1,32) = 0.30$, $p = .590$, $\eta^2 = .009$	**EtOH < H2O ($p = .002$)**
	12M	**Significant** $F(1,27) = 16.63$, $p < .001$, $\eta^2 = .381$	Not Significant $F(1,28) = .29$, $p = .594$, $\eta^2 = .011$	**EtOH < H2O ($p < .001$)**
BACE 56 kDa	6M	Not Significant $p = .135$, $\eta^2 = .064$	Not Significant $F(1,34) = 0.53$, $p = .470$, $\eta^2 = .015$	None
	9M	Not Significant $p = .875$, $\eta^2 = .001$	Not Significant $F(1,28) = 1.60$, $p = .217$, $\eta^2 = .054$	None

	12M	**Significant** $F(1,34) = 5.36$, $p = .027$, $\eta^2 = .136$	Not Significant $F(1,34) = .000$, $p = .992$, $\eta^2 = .000$	**EtOH > H2O ($p = .027$)**
BACE 70 kDa	6M	Not Significant $p = .860$, $\eta^2 = .001$	Not Significant $F(1,30) = 2.17$, $p = .151$, $\eta^2 = .068$	None
	9M	Not Significant $p = .790$, $\eta^2 = .002$	Not Significant $F(1,32) = 0.72$, $p = .404$, $\eta^2 = .022$	None
	12M	Not Significant $p = .169$, $\eta^2 = .057$	Not Significant $F(1,33) = 3.39$, $p = .074$, $\eta^2 = .093$	None
APP	6M	Not Significant $p = .451$, $\eta^2 = .015$	Not Significant $F(1,38) = 0.97$, $p = .331$, $\eta^2 = .025$	None
	9M	Not Significant $p = .367$, $\eta^2 = .023$	Not Significant $F(1,36) = 0.07$, $p = .792$, $\eta^2 = .002$	None
	12M	**Significant** $F(1,32) = 45.09$, $p < .001$, $\eta^2 = .585$	**Significant** $F(1,32) = 11.42$, $p = .002$, $\eta^2 = .263$	**Female EtOH > Female H2O ($p < .001$)** **Male EtOH > Male H2O ($p = .016$)** **Female EtOH > Male EtOH ($p < .001$)** Female H2O = Male H2O ($p = 1.000$)
Aβ	6M	Not Significant $p = .425$, $\eta^2 = .021$	Not Significant $F(1,30) = 0.01$, $p = .924$, $\eta^2 = .000$	None
	9M	Not Significant $p = .144$, $\eta^2 = .060$	Not Significant $F(1,35) = 2.14$, $p = .152$, $\eta^2 = .058$	None
	12M	**Significant** $F(1,34) = 7.99$, $p = .008$, $\eta^2 = .190$	Not Significant $F(1,34) = .66$, $p = .422$, $\eta^2 = .019$	**EtOH > H2O ($p = .008$)**

Table 5.3. Protein Expression in the Amygdala. Summary of the quantitative analysis of proteins implicated in (A) glutamate function (B) and neuropathological processes. Significant results are bolded.

Group1 mGluRs. Sex x Drinking History ANOVAs detected a significant interaction for amygdala expression of mGlu1 in 6M mice [$F(1,30) = 4.50$, $p = 0.042$, $\eta^2 = 0.130$; **Figure 5.12A**], that reflected higher mGlu1 levels in female binge-drinking mice versus their female water-drinking controls ($p = 0.001$) and male binge-drinking counterparts ($p = 0.002$). In

contrast, no significant main effects or interactions were observed for amygdala mGlu1 expression in 9M mice (see **Table 5.3A**), although a significant main Drinking History effect was detected in 12M mice [$F(1,33) = 7.93$, $p = 0.008$, $\eta^2 = 0.194$; EtOH > H2O; **Figure 5.12A"**]. No significant Sex x Drinking History interactions or main effects were detected for amygdala mGlu5 monomer expression in 6M, 9M, and 12M mice (**Table 5.3A**). In contrast, significant Sex x Drinking History interactions for mGlu5 dimer expression were detected in both the 6M [$F(1,35) = 5.94$, $p = 0.020$, $\eta^2 = .145$; **Figure 5.12B**] and 12M animals [$F(1,33) = 25.93$, $p < 0.001$, $\eta^2 = 0.440$; **Figure 5.12B"**]. In both cases, female binge-drinking mice exhibited higher mGlu5 dimer expression relative to their female water-drinking controls (6M: $p = 0.029$; 12M: $p < 0.001$) and their male binge-drinking counterparts (6M & 12M: p's < 0.001). A main Drinking Day effect was also observed for mGlu5 dimer expression in 9M mice [$F(1,34) = 4.38$, $p = 0.044$, $\eta^2 = 0.114$; EtOH < H2O; **Figure 5.12B'**].

NMDA subunits. Sex x Drinking History ANOVAs failed to detect any significant main effects or interactions for amygdala GluN1 expression in 6M, 9M, or 12M mice, or for GluN2B in 6M and 12M mice (see **Table 5.3A**). However, a significant main Drinking History effect was observed for GluN2B in 9M mice [$F(1,30) = 14.53$, $p < 0.001$, $\eta^2 = .0326$; EtOH < H2O; **Figure 5.12E'**].

Homer proteins: Sex x Drinking History ANOVAs failed to detect any significant main effects or interactions for Homer1b/c in 6M or 9M mice, or for Homer2a/b in 6M, 9M, or 12M mice (**Table 5.3A**). However, a significant Drinking History effect was observed for

amygdala Homer1b/c in 12M mice [$F(1,34) = 15.55$, $p < 0.001$, $\eta^2 = 0.314$; EtOH > H2O; **Figure 5.12F"**].

ERK. Sex x Drinking History ANOVAs indicated no significant main effects or interactions for ERK expression within the amydala of 6M or 9M mice, or for p(Tyr204)-ERK in 9M mice (**Table 5.3A**). A significant Drinking History effect on ERK was detected for the 12M mice [$F(1,28) = 12.66$, $p = 0.001$, $\eta^2 = 0.311$; EtOH < H2O; **Figure 5.12G"**]. A Drinking History effect was detected for p(Tyr204)-ERK in 6M [$F(1,38) = 14.39$, $p = 0.001$, $\eta^2 = 0.275$; EtOH > H2O; **Figure 5.12G**]. However, this group difference was reversed in the 12M mice [Drinking History effect: $F(1,28) = 9.84$, $p = 0.004$, $\eta^2 = 0.260$; EtOH < H2O; **Figure 5.12H"**].

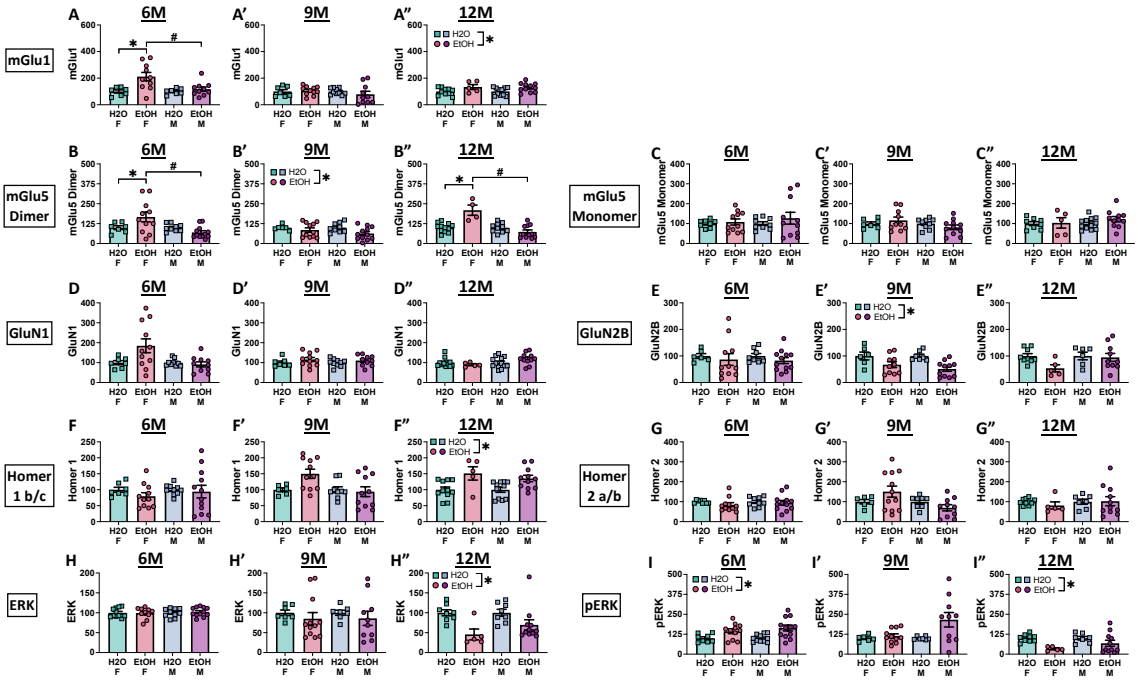


Figure 5.12. Summary of the effects of Sex and Drinking History on the expression of glutamate-related proteins in the amygdala. Group 1 mGlu receptors: (**A-A"**) mGlu1, (**B-**

B”) mGlu5 (dimer), and (**C-C”**) mGlu5 (monomer), the NMDA receptor subunits: (**D-D”**) GluN1, (**E-E”**) GluN2B, (**F-F”**) Homer 1b/c and (**G-G”**) Homer 2a/b, (**H-H”**) ERK and (**I-I”**) pERK. Data represent means ± SEMs, with specific significant interactions highlighted. Figures show means ± SEMs. *p < 0.05, EtOH vs. H20; #p < 0.05, age difference

Tau protein. A significant Drinking History effect on amygdala Tau expression was observed in 6M mice [$F(1,36) = 8.35$, $p = 0.007$, $\eta^2 = 0.188$; EtOH < H2O; **Figure 5.13A**]. Sex x Drinking History ANOVAs failed to detect any significant main effects or interactions for Tau in 9M mice (**Table 5.3B**; **Figure 5.13A’**). A main Drinking History effect was observed for Tau in 12M mice, but in contrast to 6M animals, this effect reflected higher Tau expression in binge- versus water-drinking mice [$F(1,27) = 9.30$, $p = 0.005$, $\eta^2 = 0.256$; **Figure 5.13A”**]. Sex x Drinking History ANOVAs failed to detect any significant main effects or interactions for pThr(217)-Tau in 6M, 9M, or 12M mice (all p’s > 0.051; see **Table 5.3B**). Further, no significant Sex x Drinking History interactions were detected for p(Ser396)-Tau in 6M, 9M, or 12M mice and no main effects observed for p(Ser396)-Tau expression in 6M animals (**Table 5.3B**). However, significant main Drinking History effects were observed in both the 9M [$F(1,32) = 11.53$, $p = 0.002$, $\eta^2 = 0.265$; EtOH < H2O; **Figure 5.13C’**] and 12M mice [$F(1,27) = 16.63$, $p < 0.001$, $\eta^2 = 0.381$; EtOH < H2O; **Figure 5.13C”)**.

BACE isoforms. Sex x Drinking ANOVAs failed to indicate significant main effects or interactions for BACE 56kDa in 6M and 9M mice, or for BACE 70 kDa in 6M, 9M, or 12M mice (see **Table 5.3B**). However, a significant main effect of Drinking History was observed for BACE 56kDa in 12M mice [$F(1,34) = 5.36$, $p = 0.027$, $\eta^2 = 0.136$; EtOH > H2O; **Figure 5.13D”**].

APP and AB. No significant Sex x Drinking History interactions or main effects were detected for amygdala APP expression in 6M or 9M mice (see **Table 5.3B**). In contrast, a significant Sex x Drinking History interaction was observed in the 12M mice [$F(1,32) = 11.42$, $p = 0.002$, $\eta^2 = 0.263$; **Figure 5.13F"**]. LSD tests for simple main effects indicated a higher APP expression in female binge-drinking mice, compared to both their female water-drinking controls ($p < 0.001$) and male binge-drinking counterparts ($p < 0.001$). Moreover, 12M male binge-drinking mice also displayed higher levels of APP versus their water-drinking controls ($p = 0.016$). Sex x Drinking History ANOVAs failed to indicate any significant interactions for amyloid-beta (ABeta) in 6M, 9M, or 12M mice (see **Table 5.3B**). However, a significant Drinking History effect was observed for amygdala Abeta expression in 12M mice [$F(1,34) = 7.99$, $p = 0.008$, $\eta^2 = 0.190$; EtOH > H2O; **Figure 5.13F"**].

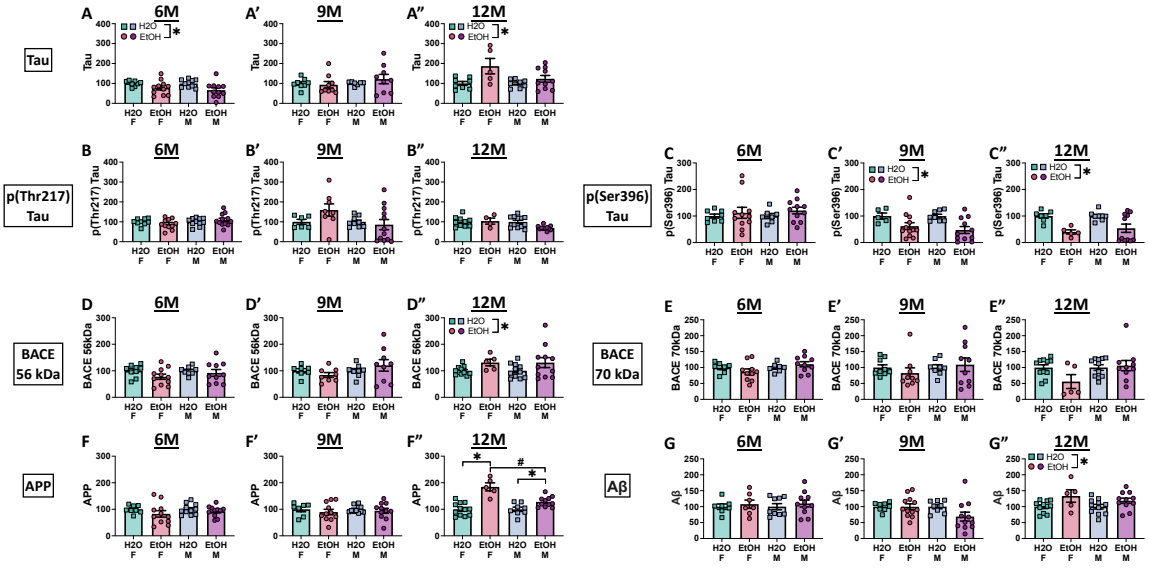


Figure 5.13. Summary of the effects of Sex and Drinking History on the expression of indices of neuropathology in the amygdala. Tau proteins: (**A-A"**) Tau, (**B-B"**) p(Thr217)-Tau, and (**C-C"**) p(Ser396)-Tau, the BACE isoforms: (**D-D"**) BACE 56kDa and (**E-E"**)

BACE 70kDa, (**F-F"**) APP (**G-G"**). Data represent means ± SEMs, with specific significant interactions highlighted. Figures show means ± SEMs. *p < 0.05, EtOH vs. H20; #p < 0.05, age difference

5.4 Discussion

A	Glutamate Related Proteins – All Brain Regions								
	Entorhinal Cortex (EC)			Prefrontal Cortex (PFC)			Amygdala (AMY)		
	6M	9M	12M	6M	9M	12M	6M	9M	12M
mGlu1	EtOH M > H2O M EtOH M > EtOH F			EtOH > H2O			EtOH F > H2O F EtOH F > EtOH M		EtOH > H2O
mGlu5 Dimer							EtOH F > H2O F EtOH F > EtOH M	H2O > EtOH	EtOH F > H2O F EtOH F > EtOH M
mGlu5 Monomer		H2O > EtOH	EtOH > H2O	EtOH > H2O	EtOH M < EtOH F EtOH M < H2O M				
GluN1			EtOH M > H2O M EtOH M > EtOH F		EtOH < H2O				
GluN2B		EtOH M < H2O M EtOH M < EtOH F	EtOH M > H2O M EtOH M > EtOH F	EtOH > H2O	EtOH F < H2O F EtOH F < EtOH M			H2O > EtOH	
Homer 1 b/c				EtOH > H2O					EtOH > H2O
Homer 2 a/b			EtOH > H2O	EtOH > H2O					
ERK			EtOH F < H2O F EtOH F < EtOH M	EtOH > H2O					H2O > EtOH
pERK		EtOH M > H2O M EtOH M > EtOH F		EtOH > H2O		EtOH F > EtOH M	EtOH > H2O		H2O > EtOH
B	**Neuropathological Protein Expression – All Brain Regions**								
Tau							H2O > EtOH		EtOH > H2O
pThr(217) Tau		EtOH M > H2O M EtOH M > EtOH F	EtOH > H2O	EtOH > H2O					
pSer(396) Tau		H2O > EtOH				H2O > EtOH		H2O > EtOH	H2O > EtOH
BACE 56 kDa	H2O > EtOH								EtOH > H2O

BACE 70 kDa	H2O > EtOH		EtOH > H2O						
APP			EtOH > H2O			EtOH > H2O			EtOH F > H2O F EtOH F > EtOH M EtOH M > H2O M
Aβ		H2O > EtOH							EtOH > H2O

Table 5.4. Comprehensive Overview of Protein Expression Variability by Age and Brain Region. Summary of the differential protein expression patterns organized by age groups across various brain regions.

The present study tracked the progression of the neurodevelopmental effects of a prior history of binge-drinking during adolescence over the normal aging process through the study of 6-, 9- and 12-month-old male and female C57BL6/J mice. This research involved the longitudinal tracking of both cognitive and affective behavior in relation to aging-related changes in NMDA and Group1 mGlu receptor-related protein expression, as well as protein indices of neuropathology associated with ADRD within the amygdala, the entorhinal cortex (EC) and prefrontal cortex (PFC). To the best of our knowledge, this study is the first to examine the long-term consequences of adolescent binge-drinking from mature adulthood into middle age in both female and male mice. Based on a relatively large literature indicating that a history of adolescent alcohol exposure is sufficient to elicit biobehavioral anomalies that can manifest in adulthood (Crews et al., 2016, 2000; Gilpin et al., 2012; Hicks et al., 2010; Pascale et al., 2022; Wooden et al., 2021), we hypothesized that binge-drinking during the period of adolescence into young adulthood would accelerate age-related decline in cognition and emotional regulation, concomitant with perturbations in glutamate-related signaling and the expression of protein markers of neuropathology. Further, based on the clinical literature demonstrating greater susceptibility to ADRD and alcohol-induced cognitive impairment in women versus men (Squeglia et al., 2009, 2011, 2012, 2014), in

addition to recent results from our laboratory indicating that female mice with a history of binge-drinking during mature adulthood exhibit more signs of cognitive impairment than their male counterparts (Jimenez Chavez et al., 2022), we hypothesized that alcohol-induced biobehavioral anomalies would have an earlier onset, occur more frequently and/or be more severe, in female versus male mice. While our results are complex, they nevertheless provide evidence supporting enduring consequences of binge-drinking during the adolescent/young adulthood period of neurodevelopment and when sex-selective effects were apparent, they manifested primarily in female subjects. Also, consistent with our study of older mice (Jimenez Chavez et al., 2022), cognition-related measures appear to be more consistently impacted by a prior history of binge-drinking than affective measures. Finally, aligning with data from studies of transgenic murine models of ADRD (Barnett et al., 2022; Hoffman et al., 2019; Kosel et al. 2020; Samaey et al., 2019), both biochemical anomalies and the expression of neuropathology markers tended to precede the manifestation of cognitive anomalies in our aging mice with a prior history of early life binge-drinking.

5.4.1. Subject factor interactions in negative affect

Girls and women are purported to exhibit greater vulnerability to, and severity of, anxiety-related disorders than boys and men (Li & Graham, 2017; Smith et al., 2022). Further, girls and women are reported to be more sensitive to the affective components of alcohol withdrawal (Varlinskaya & Spear 2015; Peltier et al., 2019). However, our prior studies of younger adult mice (aged 1.5-2.5 months) examining for sex differences in basal versus alcohol withdrawal-induced changes in negative affect yielded relatively few sex differences in the expression of anxiety-like behavior, at least in the assays employed herein

(Jimenez Chavez et al., 2020, 2022, 2023). While the results of our first sex difference study (Jimenez Chavez et al., 2020) might reflect confounds associated with exposure to pheromones of mice from the opposite sex (see Jimenez Chavez et al., 2024), our more recent reports (Jimenez Chavez et al., 2022, 2023), and the current study, assayed signs of negative affect separately in males and females to eliminate this confounding variable. Yet, despite these efforts, we detected only a few sex differences in our anxiety-related measures in studies of either 6 and 18-month-old mice (Jimenez Chavez et al., 2022) or 2 month-old mice (Jimenez Chavez et al., 2023). Moreover, in our prior study of older mice (Jimenez Chavez et al., 2022), the sex differences in affective behavior varied by age (being more prevalent in 6 versus 18 month-old mice), but did not vary as a function of prior alcohol history as the alcohol effects were observed in both male and female subjects. Consistent with this published work, the present study revealed few sex-related differences in negative affect in mice ranging from 6 to 12 months of age. Specifically, we detected an age-related reduction in the both the time spent in, as well as the number of entries into, the light side of the light-dark box test that was selective for female mice (**Figure 5.3D,G**) and 9M females buried more marbles than 3M females (**Figure 5.3K**), while males did not exhibit this age-related effect. Conversely, only males exhibited an age-related increase in the latency to first float in the forced swim test (**Figure 5.4C**), arguing that males switch their coping strategy in this assay from passive to active as they age. All three of these sex by age interactions were independent of the alcohol history of the mice.

In contrast to a relatively recent report on the long-term effects of a prior history of adolescent binge-drinking on measures of negative affect in 3xTG-AD mice (Barnett et al.,

2022), we detected only two alcohol effects with respect to our negative affect measures - a reduction in the number of light-side entries (**Figure 5.3H**) and an increase in the time spent immobile in the forced swim test (**Figure 5.4E**) – both of which were age-independent and observed in females only. While we have reported sex differences in the expression of alcohol-induced negative affect during early withdrawal (Jimenez Chavez et al., 2020, 2023), these results are our first demonstration of a female-selective, long-term, effect of prior adolescent binge-drinking history on negative affect, which likely reflects the fact that mice in the present study binge-drank throughout adolescence into young adulthood, while binge-drinking procedures were restricted in our earlier work to the 2-week period corresponding to adolescence (Jimenez Chavez et al., 2020, 2023). As the massive neuroplasticity associated with the adolescent period of development continues into early adulthood in both humans and laboratory animals (Lillard & Erisir, 2011; Larsen & Luna, 2018), it is perhaps not surprising that repeated bouts of binge-drinking during adolescence into young adulthood would have a larger or longer-lasting impact on brain function and behavior than that during adolescence alone. Indeed, we detected numerous alcohol-associated changes in protein indices of glutamate transmission and neuropathology in our aging mice with a prior binge-drinking history during adolescence/young adulthood (see **Tables 5.1-5.4**) to indicate that early life binge-drinking has long-term consequences for the brain. Further, approximately a third of these changes varied as a function of sex, with alcohol-experienced females exhibiting the most pronounced biochemical effects. Given our biochemical results, particularly those for the amygdala and PFC (see **Table 5.4**) that are key neural loci governing emotional reactivity (Koob, 2009; Salzman & Fusi, 2010, Warden et al., 2012; Wrase et al., 2008), one might question why so few alcohol-related changes in affective

behavior were apparent in the present study? Clearly, the lengthy duration of drug withdrawal is not a major factor as robust changes in brain biochemistry and neuropathology were detected at these late withdrawal time-points. Perhaps our alcohol-induced biochemical and/or neuropathological changes were simply of an insufficient magnitude and/or require more time to accumulate in brain to drive more overt changes in behavior. Related to this latter possibility, biochemical/neuropathological changes in the brain often precede behavioral anomalies in many transgenic models of neurodegenerative disease (e.g., Parkinson's and Alzheimer's Disease) (Laforet et al., 2001; Paumier et al., 2013; Tag et al., 2022), which may also hold true for the long-term consequences of early life alcohol exposure. Lastly, the possibility exists that the behavioral paradigms employed in this study to assay negative affect are not as sensitive to the long-term effects of early alcohol exposure as they are for detecting its short-term effects. Indeed, we based our decision to assay negative affect using the light-dark shuttle-box, marble-burying and forced swim tests in the present study on data collected from adolescent and young adult (primarily male) mice demonstrating that these three assays were the most reliable at detecting both the short- and longer-term (i.e., 1 versus 30 days withdrawal) effects of prior binge-drinking history (Lee et al. 2015, 2016, 2017; Szumlinski et al., 2019). However, our more recent study of 6 and 18 month-old mice detected robust alcohol effects in behavioral paradigms that we abandoned over the years that we've studied adolescent binge-drinking, including the elevated plus-maze and novel object reactivity tests (Jimenez Chavez et al., 2022). Thus, a recommendation for any future work aimed at characterizing alcohol-induced changes in the behavior of older mice is to employ a broad repertoire of assays for negative affect should they prove to be differentially sensitive to the age of the subjects tested.

5.4.2. Subject factor interactions in cognition

Aligning with our results for negative affect, we also detected relatively few age-related deficits in cognitive performance in the present study, particularly in the radial arm maze where the only significant age-related effect related to the number of working memory incorrect errors but was inconsistent over the course of maze acquisition (**Figure 5.7S**). That being said, some evidence indicated that cognitive performance varied with age and did so in a sex-selective manner. Specifically, we observed an age-dependent increase in the latency of female mice to find the flagged platform at the outset of Morris water maze testing (**Figure 5.6B**). Females also showed a significant age-dependent increase in the latency to find the hidden platform on the first day of Morris water maze training that was not as robust in male subjects (**Figure 5.6G** vs. **5.6K**). Some age-related differences in Morris water maze performance were also male-selective, including a longer latency to first enter the zone that formerly contained the hidden platform behavior by 9M males (**Figure 5.6N**) and an age-related increase in the number of entries into the former platform location during the memory probe test (**Figure 5.6Q**). However, in contrast to the data from females, these age-related effects in males are difficult to interpret as the former and latter result is indicative of poorer and better spatial memory, respectively. While only a few age-related effects were detected, such findings nevertheless align with the bulk of the extant human and laboratory animal literature arguing that females are more sensitive to normal age-related cognitive decline than males (Benice et al., 2006; Benice & Raber; 2009; Jack et al., 2015; Mielke et al, 2014; Shokouhi et al., 2020).

A prior history of early life binge-drinking also exerted few effects on cognitive performance in the Morris water maze in B6 mice. This contrasts with the results of an earlier study of 3XTg-AD mice where prior adolescent intermittent alcohol exposure (5 g/kg/day) was found to markedly enhance the cognitive impairment exhibited by these transgenic mice months later in adulthood (Barnett et al., 2022). However, it is noteworthy that the only effect observed (a reduction in the number of platform entries) was apparent in 9M females (**Figure 5.6O**), particularly considering that the alcohol-related effects on cognitive performance in the radial arm maze were most consistently expressed by female subjects (**Figure 5.7**). At the present time, it is not clear why alcohol effects apparent in 9M females [e.g., fewer entries into the former platform location (**Figure 5.6O**) and more reference memory errors towards the end of radial arm maze training (**Figure 5.7B**)] were not expressed also by the older females in this study. However, it is worth noting that 12M females with a prior history of early life binge-drinking exhibited the most consistent pattern of cognitive deficits during radial arm maze testing (**Figure 5.7**). This latter result aligns well with the extant literature indicating that females are more sensitive to alcohol-induced acceleration of cognitive decline (e.g., Fama et al., 2020; Jimenez Chavez et al., 2022; Maynard et al., 2018) and demonstrate for the first time that inbred B6 females are more vulnerable to the very long-term cognitive consequences of early life binge-drinking. An important goal of future work is to extend these findings for alcohol to even older mice (e.g., 18-24 months of age) that exhibit more signs of age-related cognitive decline upon which to assess the effects of early life binge-drinking.

5.4.3. Subject factor interactions in protein markers of glutamate transmission and neuropathology

Mentioned above, we detected a large number of alcohol-related protein changes in all three brain regions examined, despite relatively few overt changes in affective or cognitive behavior. As apparent from **Table 5.4**, the specific protein changes varied by brain region and by the age of the mice at assay, with the number of protein changes observed within the EC and amygdala increasing in an age-dependent manner, while the number of protein changes in the PFC declined with aging. Further, we failed to detect a single change in protein expression that was consistent across all age groups, even within a given brain region. It remains to be determined if that fact reflects age-related dynamics in protein expression or an artifact related to the procedural design of our study given that the tissue from 6, 9 and 12M mice were collected at different times. Nevertheless, it is clear from **Table 5.4** that the majority of alcohol-related protein changes were apparent in both sexes and that a prior history of binge-drinking during adolescence/young adulthood is not only capable of altering cellular activity (indexed by Egr-1 expression; Lee et al., 2015) and dysregulating glutamate neurotransmission in brain during early withdrawal (e.g., Lee et al., 2016, 2017, 2018b) but is sufficient to induce very long-term perturbations in cellular activity [indexed by p(Tyr204)-ERK expression] and the expression of glutamate receptor-related proteins (the vast majority of which were up-regulated during alcohol withdrawal) that are linked to neurodegenerative mechanisms, such as amyloid-beta deposition and tau hyperphosphorylation - hallmarks of both normal age-related cognitive decline (e.g., Benice et al., 2006; Fukumoto et al., 1996; Wisniewski et al., 1973) and ADRD-related behavioral

pathologies (Dodart et al., 2002; Perl, 2010; Hamley, 2012; Hersi et al., 2017; Cheignon et al., 2018; Banning et al., 2021).

Aligning with this, we detected a host of alcohol-related changes in the expression of such markers of dementia- and ADRD-associated neuropathology, including an increase in: the highly specific biomarker of AD p(Thr217)-tau, the purported early marker of AD neuropathology p(Ser396)-tau (Janelidze et al., 2020), the key component of amyloid plaques Aβ (Glenner & Wong, 1984; Chen et al., 2017; Li et al., 2023), both the 56 and 70 kD isoforms of the β-site amyloid precursor protein-cleaving enzyme BACE and its target APP (c.f., Chen et al., 2017; Li et al., 2023). These results are consistent with those from our earlier study of older B6 mice (Szumlinski et al., 2023), as well as studies of 3xTg-AD transgenic mice (Hoffman et al., 2019), in which both male and female mice with a history of alcohol-drinking exhibited comparable changes in the expression of many ADRD-associated biomarkers in brain. APP is demonstrated to directly interact with GluN2B subunits of NMDA receptors, to affect synaptic function and promote amyloidogenic fragments that exacerbate abnormal NMDA receptor activity with age (Rajão-Saraiva et al., 2023). While a causal relationship between our alcohol-induced changes in glutamate receptor expression and in the levels of neuropathology markers remain to be determined, the present results extend our earlier findings from more aged mice (Szumlinski et al., 2023) by demonstrating that a history of binge-drinking during early life is sufficient to perturb glutamate receptor expression within the EC, PFC and amygdala and to augment the expression of neuropathology markers during the normal aging process. Noteworthy is the fact that the majority of our alcohol-related protein changes were apparent in both male and female mice,

despite the fact that the behavioral effects of early life drinking were primarily sex-selective. Whether or not this brain-behavior discrepancy reflects sex differences in the behavioral manifestation of brain anomalies (i.e., the notion that males and females employ different neural mechanisms to generate behavior) and/or differential behavioral sensitivity to brain anomalies (i.e., females may be more sensitive to smaller changes in biochemistry than males) cannot be discerned from the results of the present study. Further, it is possible that a greater congruency between behavioral and biochemical outcomes might be observed in older mice that naturally exhibit higher expression of ADRD-related biomarkers (e.g., Bettio et al., 2017; Britton et al., 2022; Hendrickx et al., 2022). Nevertheless, it does appear from this collection of behavioral and immunoblotting data that, at least with respect to the proteins examined herein, the long-term biochemical effects of early life binge-drinking within the EC, PFC and amygdala do not map perfectly onto the behavior of mature adult and middle-aged mice and may in fact precede abnormal behavior.

While the majority of alcohol-related protein changes observed were not sex-selective, some sex-selectivity was noted for alcohol-related changes in protein expression that appeared to vary with the brain region examined (**Table 5.4**). For example, all but one male-selective alcohol effect on protein expression were detected in the EC, with binge-drinking males exhibiting higher mGlu1 expression at 6M, lower GluN2B at 9M, with higher GluN1, GluN2B, p(Thr217)-tau and p(Tyr204)-ERK at 12M. The only female-selective alcohol-related protein change observed in the EC was reduced ERK expression in 12M mice. The male-selectivity of the alcohol effect on protein expression within the EC aligns with the results of our recent study of more aged mice in which males exhibited a number of age

and/or alcohol-related changes in glutamate receptor expression within the hippocampus that were not apparent in female mice (Szumlinski et al., 2023). Unfortunately, we were not able to assay protein expression within the hippocampus herein. However, given that the EC is a major afferent to the hippocampus (e.g., Canto et al., 2008), we would predict similar patterns of alcohol-related changes in hippocampal protein expression and the hippocampus will be an important target of future work related to the long-term biochemical consequences of early life binge-drinking. Also consistent with our earlier report in which both male and female older mice exhibited robust alcohol-related changes in glutamate receptor expression within the PFC (Szumlinski et al., 2023), we detected many alcohol-related changes in protein expression within PFC, only a few of which were sex-selective. These included: lower mGlu5 monomer expression in 12M males, higher GluN2B in 9M females and higher p(Tyr204)-ERK in 12M. While we did not assay for amygdala protein expression in our earlier study of binge-drinking in older adult mice, a history of binge-drinking during adolescence is reported to increase AD-associated inflammation biomarkers within the amygdala of adult female, but not male, 3XTg-AD mice (Barnett et al., 2022). Consistent with a greater sensitivity of female transgenic mice to alcohol-induced neuropathology in the amygdala, we detected no male-selective alcohol-related protein changes in the amygdala while female binge-drinking mice exhibited higher expression of mGlu1 and the mGlu5 dimer at 6M, as well as higher mGlu5 dimer and APP levels at 12M. Whether and how any of our changes in protein expression drive the sex differences in alcohol-related changes in affective and cognitive function observed herein is an important research question we seek to address in future work.

5.5. Conclusions

A prior month-long history of binge-drinking during adolescence into young adulthood is sufficient to elicit changes in affect and cognition that manifest into middle age, particularly in female mice. Moreover, a prior history of binge-drinking over the period of adolescence and early young adulthood produces many biochemical changes in the EC, amygdala and PFC that are also apparent into middle age, to include increased expression of ADRD-associated biomarkers. Most of the alcohol-related changes in protein expression are not sex-selective, although male-selective protein changes were prevalent within the EC, while female-selective changes were prevalent in the amygdala. While correlational in nature, the present results add to the growing body of preclinical experimental evidence that a prior history of excessive alcohol-drinking during early life can impact brain and behavior in the very long-term in a manner that can be sex-dependent.

Chapter 6:

General Discussion

6.1 Summary of Findings

The relationship between the age of drinking onset, biological sex, and neurobehavioral outcomes constitutes a multifaceted interplay that necessitates rigorous investigation, given the considerable gaps in our current understanding. This dissertation sought to elucidate these interactions and the resulting behavioral and molecular consequences. To accomplish this, my first aim was to replicate previous findings of age-related effects of a 2-week binge drinking paradigm on negative affect observed in male mice and extend these findings to female mice (Chapter 2). While I replicated the results of prior studies indicating higher binge drinking in females versus males and in adolescents versus adults, the results from this first large-scale study indicated higher levels of negative affect in water-control mice than the binge-drinking mice on many measures, thereby complicating data interpretation. Circulating corticosterone was not correlated with the behavioral measures in this study. Furthermore, I did not replicate higher baseline anxiety-like behavior

in water-drinking adolescents versus adults, and sex differences in the expression of negative affect during early and protracted (70 days) withdrawal were minimal, which I hypothesized might reflect the concurrent behavioral testing of males and females. To address this possibility, I conducted a study examining the influence of sex-specific pheromones on marble-burying behavior of alcohol-naïve adult and adolescent mice (Chapter 3). This second study revealed interesting patterns of effects: adult male mice exhibited reduced anxiety-like behavior in the presence of female-soiled bedding, whereas adult female mice and adolescent mice of both sexes increased their marble-burying behavior in response to both male- and female-soiled bedding. Validating increased marble-burying as a behavioral sign of anxiety-like behavior, I also showed in this second study that male and female mice of both ages increase marble-burying in the presence of an innately aversive tea tree odor, which argues that the changes in marble-burying observed in the presence of sex-related chemosensory stimuli reflect the subjective state of the mouse.

Informed by the results of my second study, I overhauled how I assessed negative affect and decided to test males and females separately on different days. I hypothesized that avoiding exposure to sex-related pheromones during testing would minimize anxiety in water controls, thereby increasing the probability of detecting alcohol effects on behavior and subject-factor interactions therein. For this second large-scale study (Chapter 4), I tested for withdrawal-induced negative affect at 1 versus 30 days withdrawal to be more consistent with prior studies from the laboratory that were conducted exclusively in male or female mice. In designing this second large-scale study, I also took the opportunity to examine for the effects of binge-drinking on cognitive function, as on-going studies in the laboratory

indicated a marked sex difference in the cognitive effects of binge drinking in mature adult mice, at least in early withdrawal. I replicated higher negative affect in adult versus adolescent binge drinking mice on some behavioral measures when mice were tested in early withdrawal, indicating that testing male and female mice separately did improve the detection of age-related differences in negative affect. However, I did not detect sex differences in early withdrawal, and I did not replicate the expected age difference in anxiety-like behavior in male mice during later withdrawal, but adult-onset binge-drinking females did exhibit increased marble-burying relative to their water controls in later withdrawal – my first direct evidence of a sex difference in the persistence of withdrawal-induced anxiety-like behavior. Overall, I observed only a few alcohol-related cognitive impairments in early and later withdrawal in this study, but these were selective for the adolescent-onset binge drinking mice, with females exhibiting working and reference memory impairments in early withdrawal.

Given evidence that early-onset heavy drinking can exacerbate stressor reactivity in later life and accelerate the onset of dementia in humans, I next determined whether a more prolonged period of binge drinking throughout adolescence and into young adulthood might result in long-lasting effects on negative affect and accelerate normal age-related cognitive decline (Chapter 5). I found that adolescent binge drinking resulted in few observable long-term neurodevelopmental effects in both cognitive and affective behaviors. However, the cognitive impairments that were detected were more pronounced in female mice, who exhibited more severe deficits in cognitive function as they aged. These findings suggest a sex-specific vulnerability to the long-term neurodevelopmental impacts of adolescent alcohol

exposure. I also examined for protein correlates of behavior by examining glutamate-related protein expression and classic molecular biomarkers of neurodegeneration within the entorhinal cortex, prefrontal cortex, and amygdala. I identified numerous alcohol-related protein changes across these brain regions, indicating that a history of binge drinking during adolescence and into young adulthood has very long-term effects on brain biochemistry. Notably, age-dependent increases in protein changes were observed in the entorhinal cortex and amygdala, while the prefrontal cortex exhibited a decrease in such changes with age. Approximately a third of the molecular alterations were observed in females, correlating with the more severe cognitive decline noted in older females. These biochemical findings suggest that molecular alterations induced by early-life binge drinking may precede the onset of affective and cognitive anomalies.

6.2 The Role of Handling and Chemosensory Signals in Modulating Anxiety and Affective States During Alcohol Withdrawal in Mice

Previous work from our laboratory has consistently shown that male mice with a history of alcohol binge-drinking during the 2-week period corresponding to adolescence exhibit signs of negative affect that incubate over a 30-day alcohol withdrawal period, with no robust signs of affective disturbances at 1-day post-binge (Lee et al., 2016, 2017a). In contrast, adult male mice with a comparable 2-week history of binge drinking display immediate negative affective responses at 1 day withdrawal that dissipate by 30 days withdrawal (Lee et al., 2016, 2017a, 2018a,b). Distinct from males, the only study of binge-drinking female mice conducted by our laboratory indicated that a 2-week history of binge-drinking during either adolescence or adulthood elicits signs of negative affect at 1 day post-

binge, and some of these effects persist until 30 days withdrawal (Szumlinski et al., 2019). The apparent sex difference in the age-dependent effect of binge drinking on the expression of negative affect in early versus later withdrawal set the stage for my dissertation work. As detailed in Chapters 2 and 4, I sought to conduct direct examinations of the interaction between sex and the age of binge drinking onset on withdrawal-induced negative affect, expecting to replicate prior results for both male and female subjects at 1 and 30 days withdrawal and extend results to a more protracted withdrawal time-point (70 days). The study described in Chapter 2 also investigated changes in plasma corticosterone levels to assess the potential link between negative affect and the function of the hypothalamo-pituitary-adrenal (HPA) axis and gain a deeper understanding of the physiological mechanisms driving withdrawal-induced affective states. This is particularly important because excessive alcohol consumption is known to disrupt the HPA axis, with females exhibiting heightened stress responses compared to males. Consequently, this disruption often leads to more severe withdrawal symptoms and higher relapse rates in females (Peltier et al., 2019; Silva & Madeira, 2012; Silva et al., 2009).

Under both a 4-bottle- and 3-bottle-choice paradigm (Chapters 2 and 4, respectively), I replicated both age and sex differences in binge-drinking behavior among mice that have been reported in the literature, with adolescents drinking more than adults and females more than males (Melón et al., 2013; Moore et al., 2010). However, despite the observed differences in alcohol consumption between sexes and age, there were few notable sex- or age-related differences in the manifestation of withdrawal-induced negative affect in either study. Concerningly, we observed unusually high anxiety-like behaviors in both our

adolescent and adult water-control mice in the initial Chapter 2 study. While it is expected that adolescent water controls would exhibit higher baseline anxiety than their adult counterparts, the levels of behavior of the adult mice exceed those previously reported in the laboratory (Lee et al., 2016, 2017a,b, 2018a,b,c; Szumlinski et al., 2019), thereby masking any age-related differences in baseline anxiety. Additionally, we found no significant correlation between the expression of affective behaviors and the levels of circulating corticosterone in the initial study described in Chapter 2, which could not be readily explained (see **Table 2.1**). As I adhered to binge drinking and behavioral testing methodologies consistent with past studies (Lee et al., 2016, 2017a,b, 2018a,b,c; Szumlinski et al., 2019), I then examined other procedural factors between the study in Chapter 2 and those previous that might account for the high level of anxiety-like behavior in the water controls. Upon further examination, one procedural difference related to the differential handling experience between our binge drinking and water drinking controls. The studies in Chapters 2 and 4 employed a large number of mice, and the capacity to single-house mice in the colony room during drinking procedures was limited. Thus, in Chapter 2, the water control mice remained in their home cages in groups of 4 during drinking procedures, although their home cage was relocated to the same free-standing rack as the binge drinking mice during drinking. Consequently, the water control mice in Chapter 2 experienced considerably less handling than binge drinking mice and did not experience daily exposure to a distinct cage over the 2-week period of drinking, which would be predicted to increase their reactivity to handling and novel environments during behavioral testing. Supporting this possibility is evidence indicating that habituating laboratory rodents to regular handing

reduces aversive-like behaviors (Gouveia & Hurst, 2013; Hurst & West, 2010; Maurer et al., 2008).

Another procedural difference between the Chapter 2 study and those previous single sex studies (Lee et al., 2016, 2017a,b, 2018a,b,c; Szumlinski et al., 2019) relates to the concurrent behavioral testing of both male and female mice. Research suggests that neuronal activity within the mesocorticolimbic pathway can be modified by chemosensory signals from the opposite sex, predominantly affecting behavior through brain development processes rather than hormonal changes (Romeo et al., 1998; Bell et al., 2013a,b). This could explain why the adolescent mice in our study did not exhibit the expected incubation of withdrawal-induced anxiety-like behaviors typically observed when they are tested as adults (Lee et al., 2016, 2017a). Notably, exposure to female urinary pheromones has been shown to alleviate anxiety-like states in male mice, potentially due to changes in testosterone levels (Aikey et al., 2002). Indeed, testosterone reduces burying behavior in orchiectomized rats (Fernandez-Guasti & Martinez-Mota, 2005). Additionally, evidence suggests that chemosensory signals from sexually mature animals critically affect adolescent behavior, highlighting the influence of chemosensory stimuli during developmental periods (Zala et al., 2023). Thus, I postulated that the presence of female pheromones and/or pheromones from adult mice during our behavioral testing could have moderated the adverse effects of withdrawal in male adult and adolescent mice, respectively. While existing literature has explored the impact of female pheromones on male behavior (Fernandez-Guasti & Picazo, 1992), the influence of male pheromones on female behavioral responses remains

understudied. Addressing this significant gap, our Chapter 3 study provided a balanced investigation of pheromonal interactions across sexes and ages.

6.3 Refinement of Behavioral Assays to Account for Sex and Age Differences in Chemosensory Cue Responses

In response to the unexpected behavioral outcomes observed in Chapter 2, we hypothesized that these differences in results may arise from chemosensory stimuli present during behavioral tests. Therefore, to further investigate this hypothesis, the experiment detailed in Chapter 3 of this dissertation was conducted to examine the effects of chemosensory cues from the opposite sex on anxiety-like behaviors using the marble-burying test, a test that reliably detected both age- and alcohol-related effects in prior studies (Lee et al., 2016, 2017a). This study was comprised of multiple experiments, with the first experiment aimed at exploring the effects of sex-specific chemosensory cues, while the second study validated marble-burying as an index of anxiety-like behavior in response to aversive odor cues by comparing responses of both sexes and ages to both novel and inherently aversive odors. Our results for the first experiment in Chapter 3 provided compelling evidence that exposure to chemosensory cues shapes marble-burying behavior in a manner that is both sex- and age-dependent. Notably, adult male mice exposed to female pheromones via female-soiled bedding exhibited reduced marble-burying behaviors (**Figure 3.2C")**, an effect consistent with previous research on the anxiolytic properties of female pheromones in male rodents (Kavaliers et al., 2001). In contrast, the presence of either adult male or female odors in the bedding increased marble-burying activity in adult females and adolescents of both sexes, suggesting elevated anxiety-like behavior consistent with prior

studies of adolescent rodents exposed to odors from older counterparts (Arakawa et al., 2008; Drickamer, 1989) (**Figure 3.2C"**). To the best of our knowledge, my study examining the affective consequences of sex-related chemosensory cues in the marble burying test and the first to assay the responses of sexually naïve female subjects to such odors is the first of its kind, and the results clearly argue that sex-related chemosensory cues are potent modulators of behavior within this behavioral assay known to be sensitive to a withdrawal-induced negative affective state. Further, the results argued that my failure to detect robust age- and/or alcohol-related changes in anxiety-like behavior could very well reflect the presence of such odors during the concurrent testing of male and female subjects.

To facilitate interpretation of the effects of our sex-related chemosensory cues on marble-burying behavior, a second study examined whether exposure to a novel neutral odor (vanilla) versus a novel aversive/anxiogenic odor (tea tree oil) affects marble-burying behavior. As expected, based on evidence that tea tree oil evokes an aversive response in mice (Lustberg et al., 2020), exposure to tea tree oil consistently enhanced marble-burying across all groups when compared to both the unscented and neutral vanilla odor (**Figure 3.3A-C**), further validating marble-burying behavior as a response to an aversive state. However, it should be noted that adolescent mice also exhibited increased marble-burying in the presence of the vanilla odor, a finding not observed in adult mice (**Figure 3.3C"**). While a novel odor, vanilla is reported to be a neutral odor in adult rodents (Ueno et al., 2019; Yang & Crawley, 2009), and my marble-burying results for adult mice are consistent with such prior work and argue also that the changes in marble-burying behavior exhibited by adult mice in response to sex-specific chemosensory cues do not likely reflect their novelty. To the

best of my knowledge, no study has assessed the motivational valence of vanilla or other presumedly neutral odors (e.g., almond, lemon) in younger animals. My findings argue that while the behavior of adults is not overtly influenced by exposure to unfamiliar odors, that of adolescents is highly sensitive. Whether age-related differences in odor sensitivity within the marble-burying test extend to my other assays of negative affect or to odors associated with cleaning products or fragrances (e.g., deodorants, skin creams, perfumes, or colognes) worn by the experimenter(s) or other personnel that interact with the mice requires systematic investigation that is beyond the scope of this dissertation. Nevertheless, the marble-burying results from Chapter 3 clearly indicate that chemosensory stimuli are powerful modulators of anxiety-like behaviors that should be avoided whenever possible. Moreover, the results from Chapter 3 highlight the need to meticulously consider the complex role of environmental factors in behavioral studies involving mice, particularly those directly comparing mice of different ages and sexes, as they can mask subject-factor influences on behavior. Consequently, the study in Chapter 4 of this dissertation employed a refined methodology where male and female mice were tested separately for negative affect.

6.4 Reevaluating Negative Affect in Alcohol Withdrawal with Improved Methodologies to Reveal Age and Sex Interactions

As mentioned in Section 6.2, my second attempt to examine the interactions between sex and the age of binge drinking onset, in which males and females were tested for anxiety-like behavior on separate days to minimize exposure to chemosensory cues from the opposite sex (Chapter 4), also failed to detect consistent or robust signs of alcohol withdrawal-induced negative affect as reported by our group previously (Lee et al., 2016, 2017a,b,c; Szumlinski

et al., 2019). This being said, I did observe some interactions between Age and Drinking History in the Chapter 4 study, which were not apparent in the Chapter 2 study when males and females were tested concurrently. However, akin to Chapter 2, we detected only a few sex differences in Chapter 4, but the direction of the sex difference was not consistent across assays (i.e., females spent more time in the light side of the light-dark box than males, but females exhibited more passive coping than males in the forced swim test), and with the exception of the time spent immobile and the number of immobile episodes in the forced swim test, none of the variables associated with negative affect exhibited a significant Sex by Age by Drinking History interaction. While the combined results from Chapters 2 and 4 could be interpreted as there being little to no sex difference in the short- or longer-term effects of binge-drinking on negative affect, the simple fact that I observed so few alcohol effects and when they were observed, they were of small effect size or opposite direction compared to prior reports calls to question the reliability or robustness of the testing procedures employed. Further, while segregating males and females during testing also enabled detection of age-related differences in several of our measures, we did fail to reliably detect the expected higher indices of anxiety-like behavior in adolescent versus adult water control mice of either sex. In fact, adults buried more marbles than adolescents in the marble-burying assay, irrespective of alcohol history – a result quite opposite to what has been reliably observed in prior work. Thus, while sex-segregated testing improved the detection of certain alcohol effects, it did not improve the detection of age- or sex-specific effects.

As marked differences in the daily handling of water control mice were another potential confounding variable identified in Chapter 2 that might impact subsequent anxiety-

like behavior and mask the anxiogenic effects of alcohol withdrawal (see **Section 6.2**), attempts were made during the Chapter 4 study to enhance the handling experience of water control mice. More specifically, I implemented standardized handling across both water-drinking and binge-drinking groups throughout the 14-day drinking period, in which mice from both groups were transferred from their home cages to designated drinking cages, thereby exposing both the water controls and binge-drinking mice to a distinct environment from their home cages for 3 h/day throughout the drinking phase of the study. However, space constraints still impacted our ability to single-house the water drinking controls, and thus, the water control mice remained group-housed while in the "drinking cage," whereas binge-drinking mice were housed individually to monitor their alcohol intake. Thus, binge-drinking mice were socially isolated for 3 h/day, while the water controls were not, and the first time they were isolated from their cage mates was during the testing for negative affect. We have yet to compare the effects of daily 3-h social isolation versus no isolation on affective behavior in mice; however, prior studies conducted by the laboratory employed similar group-housing procedures for water controls and detected robust age- and alcohol-related effects on the same behavioral measures as those employed in the present study (Lee et al., 2018a,b,c; Szumlinski et al., 2019).

While the results of these aforementioned studies argue that brief, daily social isolation is not likely a key factor influencing the manifestation of baseline or withdrawal-induced changes in negative affect (Lee et al., 2018a,b,c; Szumlinski et al., 2019), I was curious to know if social versus isolated housing during the drinking period might be a contributing factor to my relatively weak effects when the experiments were conducted in the

Bio II vivarium. While not included as a chapter in this dissertation, I conducted a small follow-up study to Chapter 4 in which all mice were socially isolated (i.e., placed individually into a drinking cage) during the 3-h habituation and drinking period, and behavior was tested only on WD1 – a time when robust anxiety-like behavior should be apparent in alcohol-experienced adults (but not their adolescent counterparts) and a time when baseline anxiety-like behavior of adolescents should be higher than adults (Lee et al., 2016; Szumlinski et al., 2019). During this pilot study, I also restricted behavioral testing for negative affect to weekends, when personnel traffic and noise in the vivarium are relatively low, and all testing for negative affect was conducted exclusively by myself to minimize variability in handling techniques. Results for this study demonstrated only modest effects, with the sole age-specific finding being that adult binge-drinking mice exhibited less time spent in the light side compared to their adult water-drinking counterparts (**Figure 4.9E**). Additionally, binge-drinking animals displayed a shorter latency to bury marbles (**Figure 4.9K**). Despite all of these efforts to avoid potential confounding factors, the results from this pilot study failed to replicate prior results from single-sex studies (Lee et al., 2016, 2017a,b, 2018a,b,c). We attributed these unexpected findings in this study to inadequate power to detect robust sex or alcohol interactions, primarily due to the relatively small sample sizes (n = 6 per group). Nevertheless, these results did provide some evidence of age and sex variation in certain behaviors.

In my final chapter of this dissertation (Chapter 5), I integrated all the key insights that I had learned from my prior work as I sought to broaden our understanding of the longer-term neurobehavioral consequences of binge-drinking during early life. In Chapter 5, I

examined for changes in negative affect following a prolonged period of withdrawal by assessing behavior during mature adulthood (6-months of age) into middle age (12-months of age) (Flurkey et al., 2007). As earlier work indicated more consistent and enduring effects on withdrawal-induced negative affect in 3-month-old male mice with a 30-day versus 14-day history of binge drinking (Lee et al., 2015 vs. Lee et al., 2016), I opted to extend the duration of binge drinking from adolescence into young adulthood to impact both critical periods of brain development. Based on evidence that women are 3 times more likely to exhibit negative affect at an older age than men (Calatayud et al., 2023; Kiskac et al., 2024), I hypothesized more pronounced and earlier signs of negative affect as a result of adolescent alcohol exposure in female mice. To enhance our chances of detecting alcohol-water differences in negative affect, all mice in this study (i.e., both water- and alcohol-drinking mice) were single-housed during drinking procedures and therefore underwent identical drinking procedures with the exception of the solution consumed. Males and females were tested separately, based on the results of Chapter 4, and all testing for negative affect occurred exclusively on weekends when vivarium traffic and noise were relatively low (see below for more detailed discussion). Additionally, a single experimenter (myself) performed all behavioral assays to ensure consistency in handling and eliminate variability in experimental execution. I maintained nearly equal group sizes across different ages and sexes (n's = 11-12) to retain adequate statistical power to detect interactions with the Sex factor. Despite all of these efforts, I detected very few significant alcohol-water differences in negative affect at any of the ages tested (**Figures 5.3-5.5**).

In all likelihood, my null results for the Chapter 5 study are attributable to the extended time period between the cessation of binge-drinking and behavioral testing. Withdrawal periods varied from 5 to 11 months, far exceeding the 70-day withdrawal period employed in Chapter 2. My results from Chapter 5 argue that a prior history of binge drinking throughout adolescence and into young adulthood is not sufficient to accelerate age-related changes in negative affect. Interestingly, evidence from a longitudinal study on hippocampal recovery found that individuals diagnosed with an AUD and who exhibited behavioral deficits showed substantial improvement in hippocampal-related functions when they maintained strict abstinence from alcohol (Bartels et al., 2007). As discussed in Chapter 1, the hippocampus is key to regulating emotions and stress responses, raising the possibility that similar neuroplastic changes might happen during protracted withdrawal to restore affective behavior. Unfortunately, for practical reasons, the mice from each age group in the Chapter 5 study were studied in distinct same-age cohorts, and thus, behavioral testing for each age group was conducted in isolation from the other ages. This introduces the possibility that variations in the testing environment across the different cohorts may have influenced behavior selectively in one age group but not the others. Indeed, during the Chapter 5 study, building construction was going on both within the Bio II vivarium and in nearby facilities, and unpredictable disturbances such as loud construction noises during these prolonged withdrawal periods may have skewed our results as mice are known to be highly sensitive to these types of auditory disturbances (Konkel Neabore, 2023; Münzel et al., 2017).

At the present time, it is difficult to discern precisely why I was unable to replicate many of the Age by Drinking History interactions reported previously by the laboratory. The only remaining possibilities relate to (1) the personnel involved in conducting my studies versus those previously conducted and (2) the location of the research. Indeed, my dissertation work was conducted by myself and a relatively large team of undergraduate students (6-10 distinct students), while those previous studies were conducted primarily by a former graduate student and her long-time undergraduate assistant, both of whom were highly trained in behavioral testing, along with 3-4 additional undergraduate assistants on each experiment. Thus, differences in the experience of research assistants and the number of research assistants involved in any one project could be a contributing factor, and it is recommended for any future work that the number of research assistants involved in such large, complex, multi-factorial studies be minimized to enable more familiarization of the mice with the laboratory personnel and familiarization of the assistants with the nuances of each behavioral assay. My dissertation research was all conducted in procedural space within the Bio II vivarium, while our published work was conducted in procedural space in the Szumlinski laboratory, located outside of the Psychology vivarium. These are very distinct research environments, particularly with respect to environmental controls on noise level, concurrent research, or other activity and odor. More specifically, one procedural space in the Bio II vivarium in which the mice were tested is adjacent to the room housing the cage washer, while the other procedural space is across from the cage washer and adjacent to the laundry room. The hallway in which these two procedure rooms are located is therefore a thoroughfare for staff, with carts carrying dirty cages from both mice and rats and glass water bottles. In addition to the noise and odors associated with these carts of dirty caging, the

operation of the clothes and cage washers creates noise and vibrations, and the clothes washer emits odors from the detergent, which could all influence behavior. To mitigate the impact of vivarium noise, I conducted all of my behavioral testing for negative affect on weekends when vivarium traffic is relatively low (although the cage washer runs also on weekends). Worsening the situation, the freezer for storing cadavers is located immediately outside both procedure rooms, and the smell of cadavers is inherently aversive to rodents (Dewan et al., 2013; Hussain et al., 2013; Li et al., 2013; Kobayakawa et al., 2007). The freezer is opened on an unpredictable basis as researchers discard the cadavers of their research subjects. Further, on several occasions during my dissertation work, the bins holding the cadavers have been left out of the freezer in the hallway in front of the procedure rooms, the smell from which could be detected by a human nose even outside of the vivarium. Additionally, the colony room housing the mice is located adjacent to the staff kitchen, and smells emanating from cooked food can be detected by a human nose in the colony room. As the Psychology vivarium no longer exists and it is next to impossible to relocate the current behavioral laboratory within the Bio II vivarium, it is my recommendation that studies involving assays of negative affect be avoided as it is impossible to control for all of these environmental factors that likely impacted affective behavior in my dissertation work.

6.5 Biological Sex as a Key Variable in Alcohol-Related Cognitive Decline

Cognitive functioning encompasses a broad array of mental processes that are necessary for everyday decision-making, problem-solving, memory, and attention (Zhang, 2019). In the context of neurobehavioral research, understanding how various factors, like substance abuse, impact these functions is important, particularly as cognitive abilities

significantly influence an individual's quality of life and ability to function socially and professionally. Evidence from human studies suggests that a history of alcohol binge drinking can result in noticeable deficits in several cognitive domains, a phenomenon that is observed across the lifespan (Hiller-Sturmhöfel & Swartzwelder, 2004; Kuhns et al., 2022; Lees et al., 2020; Mota et al., 2013). Both preclinical and clinical data indicate that excessive alcohol consumption, including binge-drinking, greatly increases the risk of early onset dementia (Heymann et al., 2016; Huang et al., 2018; Ledesma et al., 2021; Piazza-Gardner et al., 2013). For one, AUDs and Alzheimer's Disease and Related Dementias (ADRDs) are highly comorbid disorders (Schwarzinger et al., 2018). Additionally, heavy alcohol consumption (> 14 drinks per week) is linked to a significant increase in the risk factors for early onset of dementia, with approximately 60% of those with early-onset dementia also diagnosed with an AUD (Hersi et al., 2017; Hoffman et al., 2019; Schwarzinger et al., 2018). A portion of my dissertation research was conducted during the 2020 coronavirus pandemic and throughout its aftermath. During this time, both the media and scientific headlines indicated that the emotional turmoil related to both the pandemic and the sociopolitical instability that followed (e.g., the attack on the US Congress and the murder of George Floyd) was associated with a marked escalation in excessive drinking. Reports stemming from the early days of the coronavirus pandemic highlighted a clear sex difference in how men and women used alcohol as a coping mechanism, with women demonstrating a strong and positive correlation between the level of psychological distress and alcohol intake (Pollard et al., 2020; Rodriguez et al., 2020). In addition to moderating affective dysfunction, biological sex is considered a key variable contributing to both the cognitive-impairing effects of alcohol (Fama et al., 2020; Maynard et al., 2018) and early-onset dementia, as well

as the heightened predisposition to alcohol-induced early-onset dementia (Ferretti et al., 2018; Hebert et al., 2013; Schwarzinger et al., 2018). Indeed, among those diagnosed with an AUD, women have an 84% increased risk of developing premature cognitive symptoms compared to a 35% increased risk in men (Peltier et al., 2019). Further, this sex difference in the cognitive consequences of excessive alcohol consumption was recapitulated in mature adult mice by the Szumlinski laboratory using the DID model of binge-drinking (Jimenez Chavez et al., 2022). Further, analytical trends over the past two decades have noted that the "gender gap" in alcohol abuse is narrowing due to a rise in alcohol abuse among females, particularly younger females (Keyes et al., 2010; White, 2020). Some researchers attribute this rise in female alcohol use to the 21st century stress epidemic identified by the World Health Organization and likely reflect the aforementioned sex differences in coping with emotional distress (Fink, 2017).

Further, studies elucidating the relationship between age of alcohol use and early-onset dementia point to an early history of alcohol abuse as the most predictive risk factor for dementia onset before the age of 65 (Hersi et al., 2017; Rehm et al., 2019; Wiegmann et al., 2020). In fact, epidemiological studies report that alcohol abuse during adolescence is the largest preventable risk factor that contributes to early-onset Alzheimer's Disease (AD) (Barnett et al., 2022; Langballe et al., 2015). While some reports indicated that adolescents and young adults decreased their alcohol intake during the first year of the coronavirus pandemic (Malta et al., 2023), other studies, including longitudinal studies, report increased drinking by youth both during the pandemic and over the years that followed (Gohari et al., 2022, 2023; McMillan et al., 2022; Meherali et al., 2021). Extensive research has shown that

an adolescent-onset of binge-drinking impacts brain maturation and can affect both emotional and cognitive processing (Chung et al., 2018; Cservenka and Brumback, 2017; Guerri & Pascual, 2010; Jones et al., 2018; Lees et al., 2020; Novier et al., 2015; Pandey et al., 2015; Tapert & Eberson-Shumate, 2022; Tetteh-Quarshie & Risher, 2023). However, very little was known at the time of this dissertation about potential sex differences moderating the relationship between the early-onset of binge-drinking and cognitive processing. Given the above data pertaining to sex differences in the cognitive consequences of excessive alcohol use, I was driven to study how a history of binge drinking earlier in life (i.e., during adolescence and early adulthood) might differentially impact cognitive function in male versus female subjects. As such, in Chapters 4 and 5 of my dissertation, I also tested mice in two well-established cognitive assays employed by the Szumlinski laboratory: the Morris water maze (MWM) and the water version of the radial arm maze (RAM) (Ary et al., 2013; Datko et al., 2017; Denning et al., 2024; Jimenez Chavez et al., 2022; Lominac et al., 2005; Szumlinski et al., 2023a, 2023b). The MWM is a classic paradigm used to assess spatial learning and memory (Vorhees & Williams, 2006), in which mice were trained to locate a hidden platform in a water tank using visual cues. The water version of the RAM is used to assay working and reference memory in which mice are trained to navigate an eight-arm maze with four arms containing hidden escape platforms, which are removed as they are located. Thus, on each trial, the mice have to remember which arm was recently visited and which contained or did not contain an escape platform, with working memory load increasing across the 4 trials (Penley et al., 2013). Recently, we have modified our MWM procedures to include a test for reversal learning (Jimenez Chavez et al., 2022), which is particularly dependent upon intact prefrontal cortex function (de Bruin et al., 1994), to probe for alcohol-

related deficits in this cognitive domain. The water version of the RAM was adopted by the Szumlinski laboratory in lieu of the more traditional RAM procedure in which mice seek out a palatable food reward in 4 of the arms to avoid potential interpretational confounds associated with food restriction as well as potential age- and sex-related differences in food motivation (Lominac et al., 2005). Based on existing human (Cservenka & Brumback, 2017; Huang et al., 2018; Ledesma et al., 2021; Squeglia et al., 2009, 2011; Norman et al., 2011; Novier et al., 2015) and rodent literature (Grifasi et al., 2019; Hoffman et al., 2019; Jimenez Chavez et al., 2022; Salling et al., 2016; Van Hees et al., 2022) indicating that heavy drinking can enhance cognitive decline, in addition to limited human evidence that alcohol-related neurocognitive anomalies tend to be more pronounced in adolescent females than males (Squeglia et al., 2009, 2011), I hypothesized in both Chapters 4 and 5 that a history of binge drinking will induce and/or accelerate cognitive decline and associated neuropathology, with adolescent females particularly affected.

In my Chapter 4 study, adolescent and adult mice underwent the 2-week binge drinking protocol, and mice were assayed for cognitive impairment over the weeks that followed testing for negative affect on day 1 or 30 of alcohol withdrawal, with the intent to capture both early and more protracted effects of binge drinking. However, in contrast to the results of a prior binge drinking study of older mice (Jimenez Chavez et al., 2022), the Chapter 4 study detected alcohol-dependent effects for only one variable under MWM procedures. These effects were apparent for the number of entries into the former platform location during the memory probe test (a measure of long-term spatial recall; **Figure 4.6N**) and were interpreted as reflecting poorer spatial recall by adolescent-onset binge-drinkers but

better spatial recall by adult-onset binge-drinkers, relative to their water-drinking controls. Although the female adolescent mice consumed the most alcohol and exhibited the highest BAC (**Figure 4.2A**), we detected no sex differences in this or any other measure during MWM testing. However, consistent with the results of a study in older mice (Jimenez Chavez et al., 2022), more variables were affected by prior drinking history under RAM procedures in Chapter 4 (**Figure 4.7**), suggesting that working and reference memory may be more vulnerable to alcohol than spatial learning and memory. However, unlike our results for older mice (Jimenez Chavez et al., 2022), the alcohol-related cognitive deficits exhibited by adolescent- and adult-onset binge-drinking mice in Chapter 4 were not consistent across variables, with some variables exhibiting alcohol effects for only 1-2 days during early learning, while others emerged in the middle of the first week of training but dissipated quickly thereafter (**Table 4.6**). Notably, an examination of the change in both working memory correct (**Figure 4.7S**) and incorrect errors (**Figure 4.7D'**) suggests that a history of binge drinking impairs between-session learning in the RAM, irrespective of sex and age of onset. This study is the first to assess the impact of adolescent and early adult binge drinking on RAM performance, and it is possible that a 2-week history of binge drinking under our DID procedures is insufficient to induce significant cognitive dysfunction in either the short- or long-term. Supporting this possibility, alcohol-related cognitive deficits (and sex differences therein) were apparent in older mice (6-18 months) with a 1-month history of binge-drinking (Jimenez Chavez et al., 2022). This raises the possibility that the relatively weak cognitive effects observed in Chapter 4 reflect the subchronic nature of the binge-drinking experience. Thus, although evidence indicates that non-dependence drinking can alter the expression of AD-related genes in both adolescent and adult B6 mice (Hoffman et

al., 2019; Salling et al., 2016), a more prolonged alcohol-drinking history may be required to induce robust cognitive effects in younger mice.

Thus, in Chapter 5 of this dissertation, I extended the period of binge drinking from early adolescence into young adulthood, with the hypothesis that a 1-month period of binge drinking throughout these key periods of neurodevelopment and neuro-refinement might be sufficient to accelerate the onset of cognitive decline. In contrast to the studies described in Chapters 2-4, I examined for alcohol-related cognitive effects during older age, specifically from mature adulthood (6 months old; 6M) through to middle age (12 months old; 12M; Flurkey et al., 2007). In retrospect, it may have been more logical to examine the cognitive consequences of a 1-month binge drinking period in mice of comparable ages to those employed in Chapters 2-4 of this dissertation to confirm whether or not increasing the chronicity of binge drinking does in fact augment signs of cognitive impairment. However, at the time, I was driven to examine the longer-term consequences of adolescent binge drinking based on the aforementioned human literature indicating that an early history of alcohol abuse is the most predictive and preventable risk factor for early dementia onset (Barnett et al., 2022; Hersi et al., 2017; Langballe et al., 2015; Rehm et al., 2019; Wiegmann et al., 2020) and the simple fact that no preclinical animal had attempted to recapitulate this phenomenon under highly relevant binge drinking procedures.

While procedural differences related to the duration of alcohol intake and the age of mice at cognitive testing existed between the studies in Chapters 4 and 5, it was interesting to note that behavior under MWM procedures was less impacted by prior binge drinking history

than behavior under RAM procedures. Studies using diverse ethanol delivery methods such as intragastric administration and ethanol vapor exposure, which produce high binge-level BACs, show that adolescent ethanol exposure leads to both acute and long-term cognitive impacts, as evidenced by MWM performance, especially in tasks requiring cognitive flexibility (Chin et al., 2011; Coleman et al., 2011; Obernier et al., 2002; Schulteis et al., 2008). This suggests that cognitive deficits from adolescent ethanol exposure are proportional to ethanol exposure levels, as evidenced by higher BACs in these studies than in our research on voluntary binge drinking, suggesting that MWM procedures might be less responsive than RAM in identifying cognitive impairments associated with voluntary binge drinking (Jimenez Chavez et al., 2022). In Chapter 5, the only alcohol-related effect observed under MWM procedures was a lower number of platform entries during the memory probe test (the same variable impacted by alcohol in Chapter 4), but this effect was observed in 9M-old female mice only (**Figure 5.6O**). Interestingly, these same 9M-old females exhibited more reference memory errors than their water controls over several days of RAM maze training (**Figure 5.7B**). Curiously, alcohol affected neither of these measures in the older 12M-old females, although binge drinking females tested at 12M exhibited the most consistent pattern of alcohol-related cognitive deficits during RAM testing (**Figure 5.7**). Together, the results from Chapter 5 align with the existing literature indicating that females are more sensitive to alcohol-induced acceleration of cognitive decline (Fama et al., 2020; Jimenez Chavez et al., 2022; Maynard et al., 2018). Whether or not alcohol effects would manifest in males and be more pronounced or earlier in onset in females if mice were allowed to binge drink for a longer period than 2 h each day is beyond the scope of this dissertation. Supporting this possibility is evidence that just 10 days of binge-drinking during

adolescence under 4-h DID procedures (which doubles the alcohol intake of the mice) is sufficient to induce a deficit in novel object recognition when assessed at 40-days withdrawal (Van Hess et al., 2022). Nevertheless, the results from Chapter 5 demonstrate for the first time that a history of binge drinking during adolescence and into young adulthood produces some very enduring effects on cognitive function in inbred B6 mice that manifest selectively in females, providing a framework upon which to expand our understanding of the long-term impact of early-life heavy drinking on brain and behavior.

6.6 A History of Adolescent Binge Drinking Alters Protein Expression in the Entorhinal Cortex, Prefrontal Cortex, and Amygdala in the Long-Term

The final aim of my dissertation was to relate the behavioral anomalies observed in Chapter 5 to the expression of classic molecular biomarkers of glutamate transmission and neuropathology. The original experimental design of the biochemical aspect of the Chapter 5 study was to assay for group differences in protein expression within the entorhinal cortex (EC), prefrontal cortex (PFC), and amygdala (AMY) in-house using immunoblotting, while the hippocampal tissue was to be shipped to our collaborator, Dr. Ramon Valezquez at Arizona State University, for assay of multiple ADRD-related markers, including 3-4 different forms of phospho-tau, using his well-established ELISA assay. Regrettably, the hippocampal samples were compromised during shipping, resulting in protein degradation and the inability to conduct the ELISA, despite the best efforts by the Valezquez laboratory. Nonetheless, I was successful in immunoblotting procedures on the remaining three brain regions. As detailed in Chapter 5, we identified numerous alcohol-related protein alterations within the EC, PFC, and AMY (**Table 5.4**), despite detecting relatively few changes in

affective or cognitive behavior. Further, an examination of these protein changes revealed regionally distinct, age-related patterns in protein expression, with the EC and AMY exhibiting an age-dependent increase in the number of protein changes and the PFC exhibiting an age-related decline. Such findings suggest that different brain regions possess distinct susceptibilities to the long-term effects of alcohol, likely influenced by their developmental trajectories and specific functions (Lannoy & Sullivan, 2021). Of note, however, no single protein change was consistently observed across all ages within any brain region. As the 6M, 9M, and 12M mice were tested for behavior in distinct same-age cohorts, the brain tissue was also collected at different times. Thus, at present, it is difficult to discern whether the apparent age-dependent changes in the effects of alcohol on protein expression (or the lack of a consistent/persistent alcohol effect across all ages) reflect the genuinely complex long-term effect of adolescent binge-drinking on protein expression or merely the fact that the tissue was collected at distinct times over the course of a year. While I cannot discern between these possibilities at present, the immunoblotting data from Chapter 5 indicate that one-third of the observed alcohol-related protein changes were sex-selective, which cannot be readily accounted for by differential environmental conditions across cohorts as tissue was collected from the females and males within each age group at the same time. To the best of my knowledge, the Chapter 5 study is the first large-scale examination of the very long-term effects of adolescent binge drinking history on glutamate receptor expression and ADRD-related neuropathology markers in the brain, and the data provide convincing evidence that our binge drinking procedure was sufficient to induce a large number of protein changes in later life, a proportion of which are sex-selective.

Noteworthy is the observation that the mice in Chapter 5 exhibited increases in the majority of ADRD-related biomarkers examined herein, including phosphorylated tau, β-site amyloid precursor protein-cleaving enzyme (BACE), its target amyloid precursor protein (APP), and presumably resultant β-amyloid peptides. Such data indicates that early-life binge drinking can induce neuropathological changes in the brains of mature and middle-aged adults that are similar to those seen in much later stages of the normal aging process or in those with ADRDs. My findings align with previous research indicating that early exposure to alcohol can have long-lasting impacts on brain health, potentially accelerating neurodegenerative disease processes (Chen et al., 2017; Glenner & Wong, 1984; Janelidze et al., 2020; Li et al., 2023). Tau proteins are essential for stabilizing microtubules in neurons, but hyperphosphorylation of tau can lead to the formation of neurofibrillary tangles (NFTs), which are hallmark features of Alzheimer's disease (Medeiros et al., 2011). Early alcohol exposure has been shown to increase tau phosphorylation, which disrupts microtubule stability and impairs axonal transport, contributing to neurodegeneration (Barnett et al., 2022). The elevation of specific phosphorylated tau epitopes, such as p(Thr217)-tau and p(Ser396)-tau, observed in my Chapter 5 study emphasizes the potential for early life exposure to alcohol to precipitate tau pathology at later developmental stages not normally associated with signs of neuropathology and neurodegeneration. Amyloid plaques, primarily composed of β-amyloid peptides, are integral to AD pathology (Chen et al., 2017; Hampel & Shen, 2009; Li et al., 2023; Zhang et al., 2021). These peptides result from the cleavage of APP by BACE1 and γ-secretase. My immunoblotting results in Chapter 5 suggest a heightened amyloidogenic processing of APP, potentially leading to the formation of amyloid plaques, a hallmark of AD (O'Brien & Wong, 2011; Orobets & Karamyshev, 2023).

The increase in both the 56 and 70 kD isoforms of BACE in our study of the long-term effects of adolescent binge drinking in Chapter 5 is consistent with the short-term effects of binge-drinking during later life on BACE protein expression within the PFC and hippocampus reported previously by our group (Szumlinski et al., 2023a), as well as the results of other studies indicating that excessive alcohol intake can upregulate BACE1 activity in the hippocampus, striatum, and cerebellum, thereby increasing the production of β-amyloid (Gong et al., 2021; Kim et al., 2011; Yussof et al., 2020). Further, APP is also known to directly interact with the GluN2B subunits of NMDA glutamate receptors, which are important for synaptic plasticity and cognitive function (Rajão-Saraiva et al., 2023) and are upregulated in my immunoblotting study by a prior history of binge-drinking during adolescence. The interaction between APP and GluN2B has significant implications for synaptic function, as it promotes the generation of amyloidogenic fragments that exacerbate abnormal NMDA receptor activity, particularly with age. This abnormal activity can lead to synaptic dysfunction and neurodegeneration, other key features of ADRD (Rajão-Saraiva et al., 2023; Zhang et al., 2023). Together, this collection of findings indicates that alcohol consumption may enhance amyloidogenic processing of APP, thus promoting plaque formation and subsequent neurodegeneration both in the short (Szumlinski et al., 2023a) and very long term (Chapter 5).

As mentioned above, approximately one-third of our alcohol-related protein changes were sex-selective (**Table 5.4**). Notably, the EC was the primary region exhibiting male-selective effects, with binge-drinking males exhibiting increased mGlu1 expression at 6M, decreased GluN2B levels at 9M, and elevated expression of GluN1, GluN2B, p(Thr217)-tau,

and p(Tyr204)-ERK at 12M. Conversely, the sole female-selective change observed in the EC was a reduction in ERK expression at 12M. The EC serves as a critical hub for the relay of sensory and cognitive information to the hippocampus, which is essential for processes such as memory formation and spatial navigation (Canto et al., 2008; Hyman et al., 1986; Ibáñez et al., 1995; López-Madrona & Canals, 2021; Zhang et al., 2013). The male-selective alcohol effects on protein expression within the EC observed in Chapter 5 are consistent with prior research indicating that many of the age- and alcohol-related changes in glutamate receptor expression within the hippocampus reported in the literature are male-selective effects of sex differences in alcohol-related changes in glutamate receptor expression (Akkus et al., 2018; Carzoli et al., 2019; Giacometti & Barker, 2020; Szumlinski et al., 2023a). Given the functional interrelation between the EC and hippocampus, it has been proposed that alcohol-induced alterations in the EC are likely to influence hippocampal function and protein expression (Crews et al., 2000) and ultimately influence behavior. However, it would appear based on the collective behavioral and immunoblotting results from the Chapter 5 study that these male-selective changes in glutamate receptor expression and neuropathology markers within the EC were insufficient to impact cognitive function, as no male-selective effects of alcohol were detected for our cognitive measures. Nevertheless, they do not preclude the possibility that any of the observed protein changes within the EC may "set the stage" for greater vulnerability to cognitive decline or neuropathology beyond middle age – a possibility that could be tested in future studies of more aged mice.

Our prior report on the effects of binge-drinking during later life revealed many changes in glutamate receptor expression within the PFC of both male and female mice

(Szumlinski et al., 2023a). Consistent, in part, with these data, I detected numerous alcohol-induced alterations in protein expression within the PFC in the Chapter 5 study, of which only a few were sex-selective. Specifically, lower mGlu5 monomer expression was observed in 12M males, higher GluN2B levels in 9M females, and higher p(Tyr204)-ERK levels in 12M mice. The PFC is key for executive functions, decision-making, working memory, and impulse control, and alterations in glutamate receptor expression and cellular activity in this region can have profound effects on cognition and behavior (Buard et al., 2022; Rmus et al., 2023). Given that we only detected long-term behavioral effects on working memory measures in female subjects, it is difficult to reconcile the very few sex differences in alcohol-related changes in protein expression within PFC with the female-selective behavioral anomalies in Chapter 5. However, it is noteworthy that the elevated GluN2B levels in females may reflect an upregulation of NMDA receptor activity, potentially associated with higher excitotoxicity (Xu et al., 2023), which might accelerate cognitive decline. Indeed, the elevated p(Tyr204)-ERK expression in alcohol-experienced 12M females argues for greater PFC cellular excitability, which aligns with prior studies indicating that alcohol exposure can modulate ERK activity to drive maladaptive neural responses (Agoglia et al., 2015; Ron & Messing, 2013).

The amygdala is a critical brain region involved in emotional regulation and stress responses, and its dysfunction is implicated in various neuropsychiatric and neurodegenerative disorders (Gonzalez-Rodriguez et al., 2023; McGaugh, 2004). Alcohol-induced alterations in the amygdala can thus have significant implications for affective and cognitive functions. Studies have shown that chronic alcohol exposure can lead to increased

neuroinflammation, oxidative stress, and synaptic dysfunction in the amygdala, contributing to behavioral and cognitive impairments (Abrahao et al., 2017; Götz et al., 2001; Mineur et al., 2022). Although we did not analyze amygdala protein expression in our previous study of binge-drinking older adult mice (Szumlinski et al., 2023a), adolescent binge drinking elevates AD-related inflammation biomarkers in the amygdala of adult female, but not male, 3XTg-AD mice (Barnett et al., 2022). This observation in a transgenic model of ADRD aligns, in part, with the observation from Chapter 5 that none of the alcohol-related protein changes observed in the amygdala were male-selective. In contrast, female binge-drinking mice exhibited higher expression of mGlu1 and the mGlu5 dimer at 6M, as well as elevated levels of the mGlu5 dimer and APP at 12M. The higher expression of mGlu1 and mGlu5 dimers in female mice suggests an upregulation of metabotropic glutamate receptor signaling, which would be predicted to enhance excitatory neurotransmission. This upregulation may contribute to heightened vulnerability to alcohol-induced neuropathology and cognitive deficits in females (Giacometti & Barker, 2020; Wang et al., 2015). Curiously, evidence suggests that the activation of Group 1 mGluRs can facilitate nonamyloidogenic processing of APP by α-secretase, potentially protecting from Aβ-mediated neurotoxicity (Jolly-Tornetta et al., 1998; Meziane et al., 1998). Whether the concomitant increase in the mGlu5 dimer and elevated expression of APP within the amygdala of alcohol-experienced 12M females reflect some interaction between these proteins remains to be determined. However, such an interaction does not readily explain the facts that (1) alcohol-experienced 12M males and females exhibited higher Aβ expression within the amygdala and (2) only the 12M females exhibited working memory impairments.

Evidence suggests that excessive drinking during adolescence disrupts glutamate receptor expression in key brain regions, including the EC (Cippitelli et al., 2010), PFC, (Sircar & Sircar, 2006; Pascual et al., 2009; Arce et al., 2023), and AMY (Roberto et al., 2003, 2004, 2006, 2012). Taken together, our results demonstrate that even a 1-month-long history of binge-drinking during adolescence extending into young adulthood is sufficient to cause lasting changes in glutamate receptor and markers of ADRD-related neuropathology within the EC, PFC, and AMY that are evident into middle age, highlighting the very long-term impact of early-life alcohol exposure on the brain. As a number of these protein changes were apparent at 6 months of age, while behavioral anomalies were not detected until 9 or 12 months of age, these protein changes preceded behavioral anomalies, which is a finding consistent with the timelines of neuropathology versus behavioral anomalies in many transgenic mouse models of ADRD (see https://www.alzforum.org/research-models/alzheimers-disease/commonly-used). A key goal for future work will be to replicate these findings and extend them to older ages to determine whether the observed changes in protein expression do accelerate the onset of cognitive decline more selectively in females, as suggested by the results of Chapter 5.

6.7 General Limitations & Future Directions

As highlighted throughout this dissertation, I encountered significant challenges in replicating the earlier findings from single-sex studies conducted by our laboratory (Lee et al., 2016, 2017a,b, 2018a,b,c; Szumlinski et al., 2019), despite my best efforts to identify and address procedural variables that might account for this failure to replicate. In the end, a key factor likely contributing to my null results relates to the physical setting in which my studies

took place due to the aforementioned issues with noise, vibration, and odors that were beyond my control. Unfortunately, merely conducting studies during the quietest times of the week to minimize exposure to these stressors was still not successful at bringing out group differences in behavior. One recommendation that I offer should this line of research continue is to install sound-attenuating insulation and perhaps employ background white noise to mitigate the impact of outside noise on behavior. I also strongly encourage the relocation of the carcass freezer away from procedural and colony space to minimize exposure to highly aversive and stress-provoking odors. While I cannot know for certain whether masking external noise and removing cadaver-related odors will necessarily increase the reliability of our assays for detecting age- and alcohol-related effects on our measures of negative affect, these adjustments are not predicted to worsen study outcomes.

Another notable limitation of this dissertation is the restriction of the mice to a 2-hour drinking period. The decision to employ a 2-h drinking paradigm was based on the definition stipulated by the National Institute on Alcohol Abuse and Alcoholism (NIAAA), which defines binge drinking as achieving a blood alcohol concentration (BAC) of 0.08 g/dL within approximately 2 hours (NIAAA, 2004). However, preclinical studies suggest that extending the period of voluntary binge-drinking to 4 hours can result in more consistent and pronounced signs of negative affect following a history of binge drinking (Younis et al., 2019), with these aversive behavioral manifestations persisting even 40 days post-binge (Van Hees et al., 2022). This discrepancy underscores that the 2-hour period may not fully encapsulate the complexities of binge drinking behaviors observed in humans, where individuals rarely limit their intake of a 2-h period.

It is important to recognize that the current definition of binge drinking was established by the NIAAA in 2004. Over the past two decades, rodent biomedical research has evolved significantly, necessitating a re-evaluation of our experimental models. Acknowledging that clinical definitions may not always translate directly to rodent models is important for enhancing the translational relevance of preclinical studies. By refining experimental protocols to better capture the nuanced drinking behaviors characteristic of human binge drinking, we can improve the ecological validity of these models in representing the associated neurobiological and behavioral consequences. Moreover, the relatively few behavioral signs observed following a history of binge drinking in this dissertation may be attributed to the limitations of restricting the drinking period to 2 hours. This aligns with the understanding that mild and moderate alcohol use disorder (AUD) typically exhibits a lower rate of psychiatric comorbidities compared to the more severe diagnoses (Helle et al., 2020; Ravikanth & Sultan, 2020). This relationship suggests that the severity of AUD is positively correlated with the likelihood of co-occurring psychiatric conditions, such as depression and anxiety. Nevertheless, the findings from my dissertation work suggest that even a subchronic history of relatively brief bouts of binge-drinking is sufficient to induce both short-term and very long-term effects on the brain and behavior.

The data from my dissertation was also highly variable, resulting in the omission of statistical outliers and, for some variables, largely uneven sample sizes. Given the large scale of these studies, increasing the number of mice per group would be difficult to accomplish logistically due to resource constraints. While identifying the external factors contributing to

this variability is a top priority (e.g., environmental noise and odors), future studies could also focus on standardizing experimental protocols, personnel training, and environments for housing and testing the animals and increasing the uniformity in the timing and conditions of experiments to help reduce variability. Additionally, the high number of analyses performed raises the possibility that some of the results, particularly for the immunoblotting, may have reflected Type I errors. To mitigate this risk, future research should consider employing advanced statistical techniques. For example, path analyses can help by identifying and modeling complex relationships between variables, providing a clearer understanding of causal pathways (Bick et al., 2021). Meta-analytical approaches can aggregate findings across multiple studies, increasing statistical power and providing more robust estimates of effect sizes (Conn et al., 2012). Employing robust statistical methods, such as multilevel modeling, Bayesian approaches, or robust ANOVAs, can handle high variability and uneven sample sizes more effectively (Gordon, 2019; Kruschke & Liddell, 2018). These approaches are encouraged in future work to enhance the reliability and validity of the findings. Related to the physical laboratory space, it is entirely possible that while the Light-Dark Box, Marble Burying, and Forced Swim Test were the most alcohol-sensitive assays in the Psychology laboratory space (Lee et al., 2015, 2016, 2017a), it is entirely possible that the alcohol sensitivity of our different behavioral assays for negative affect is different in the Bio II space. Indeed, binge drinking effects were detected on the behavior of older mice in the Elevated Plus Maze and Novel Object Reactivity tests when conducted in Bio II (Jimenez Chavez et al., 2022), while alcohol induced negative affect was not reliable observed in our prior studies conducted in the Psychology laboratory (Lee et al., 2015, 2016, 2017a). Additionally, the acute nature of the stressors employed in these studies may only partially

capture the potential for behavioral sensitization observed with repeated stress exposure. Repeated exposure to stressors is well-characterized in the literature as a factor that can sensitize behavior (Greenwood et al., 2014; Sánchez-Marín; 2022). Therefore, future research should investigate how a prior history of binge drinking, particularly during adolescence, might influence the development or magnitude of the behavioral response to repeated stress. This approach could provide deeper insights into the long-term neurobiological and behavioral impacts of alcohol exposure. Thus, it may be worthwhile to return to a more comprehensive behavioral screen for negative affect in order to increase the scientific rigor of future work.

With respect to tests of cognitive function, it may be beneficial for future research to consider employing so-called "land-based" behavioral assays to avoid the potential confound of stress induced by forced swimming that occurs in the MWM (Shoji et al., 2016) and the water version of the RAM. Indeed, evidence suggests that the cognitive performance of younger mice is more susceptible to swim stress during MWM testing than that of older mice (Brito et al., 2023), and this likely generalizes to the water version of the RAM. Thus, while we did detect some signs of alcohol-induced cognitive impairment in MWM and RAM, these alcohol effects, as well as potential age- and sex-related effects, are predicted to be more pronounced in the absence of overt swim stress. To this end, the Barnes Maze has proven to be very effective for assessing impairments in spatial cognition in younger mice (Brito et al., 2023; Gawel et al., 2019), while the land version of the RAM or the Y-maze could be employed to assay working memory. The Y maze could also be employed to assay reversal learning. While these land paradigms to assess working memory require food restriction

(which is a stressor; Fu et al., 2017) and employ palatable foods to motivate behavior, which may vary in an age- and sex-dependent manner (Freeman et al., 2021; Friemel et al., 2010), these paradigms avoid daily exposure to a potent physical and psychological stressor. Mice will readily exhibit operant-conditioning for liquid sucrose reinforcement under operant-conditioning procedures without the need for fluid or food restriction, and there exist a number of cognitive assays that employ operant conditioning procedures to examine for basic operant learning, attention, impulsivity, and cognitive flexibility (e.g., 5-choice serial reaction time task) that do require considerably more training than MWM or RAM to acquire in mice but are not confounded by overt stressors.

In the context of our longitudinal study, an important goal of future work is to extend these findings to even older mice that exhibit more signs of age-related cognitive decline to assess the effects of adolescent binge-drinking. Given that the majority of our cognitive results were evident at 12 months, particularly in female mice, future studies should consider starting with 12-month-old mice and extending the age range of mice tested to 18-24 months of age. Another procedural variable that might be considered for future work to augment the chances of detecting binge drinking effects on affect and cognition is to increase the amount of time mice binge-drink each day. To advance our neurobiological understanding of alcohol-induced neuropathology, future studies could employ immunohistochemistry techniques to complement the findings derived from my immunoblotting studies. While immunoblotting has proven invaluable for detecting specific protein expression levels within samples, it lacks the spatial context necessary to understand the distribution and localization of these proteins within intact tissues (Ghosh et al., 2014). Immunohistochemistry, on the

other hand, can provide this key spatial information, offering insights into the precise anatomical and cellular localization of protein alterations (Duraiyan et al., 2012). Future research can leverage the immunoblotting data from this dissertation to inform and enhance immunohistochemical analyses. First, immunoblotting can identify differentially expressed proteins in response to alcohol exposure in specific brain regions. Subsequent immunohistochemical studies can then elucidate the cellular distribution and cell specificity of the observed protein changes, which are relevant to elucidating the neurocircuits affected by alcohol or involved in alcohol-induced behavioral anomalies.

In sum, while my dissertation research had its limitations, the results obtained laid the foundation and provided many recommendations for follow-up studies examining both the short- and long-term consequences of binge-drinking on the brain and behavior, and sex differences therein.

6.8 Conclusion

In conclusion, my dissertation work highlights the complex relationships between a history binge drinking, age of drinking onset, biological sex, and their related biobehavioral consequences. These findings emphasize the need to consider both biological sex and age when evaluating both the immediate and prolonged effects of binge-drinking in relation to the etiology of AUD and dementia. My dissertation work also informs as to the role of a number of procedural variables in regulating affective behavior and how this regulation is both sex- and age-dependent, even in the absence of alcohol. These procedural variables are important considerations for the manifestation of both the short- and longer-term behavioral

effects of binge-drinking in laboratory animal models, which is critical for our fundamental understanding of alcohol's impact on the brain. The recommendations for future work provided in this dissertation should enhance the accuracy and consistency of future studies, enabling a deeper understanding of alcohol's nuanced effects on behavior and brain function.

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