ABSTRACT

Age-Related Differences in Ethanol Withdrawal,
Withdrawal-Induced Anxiety, Cognition, and Response to Allopregnanolone

Adelle Novier, Ph.D.

Mentors: Jaime L. Diaz-Granados, Ph.D.
Douglas B. Matthews, Ph.D.

Research with humans and rodents indicates that symptoms of alcohol withdrawal may be more severe in the elderly compared with younger adults. However, investigations of the effects of repeated ethanol intoxications and withdrawals on persistent deficits in motor and cognitive performance in aged rats are limited. The present study thus sought to investigate age-related differences in the severity of ethanol withdrawal in adult and aged rats and examine potential long-term deficits resulting from chronic liquid ethanol diet. Results revealed that adult rats exhibited robust signs of ethanol withdrawal using a four-item behavioral scale (rigidity, hypoactivity, irritability, and intentional tremors) but showed only a non-significant trend of anxiety-like behavior and hyperactivity in the elevated plus maze and open field tasks during withdrawal. In contrast, aged animals consumed significantly less ethanol per body weight than adults and achieved minimal withdrawal scores using the standardized scale, but exhibited profound anxiety-like behavior during withdrawal. After a 14 day cessation from ethanol, adult and aged rats treated with chronic ethanol diet showed spatial learning deficits in

water maze acquisition and a cognitively-challenging reversal paradigm. Chronic ethanol consumption did not result in significant motor impairments in either age group as assessed by the aerial righting reflex. When challenged with an acute dose of ethanol, adult and aged animals demonstrated metabolic but not cognitive tolerance to ethanol. Finally, we investigated the effects of an acute dose of allopregnanolone on spatial memory in aged rats. In accordance with previous research, adult animals showed impaired performance when treated with allopregnanolone. However, the results for the aged animals were not statistically significant. Interestingly, animals receiving an acute ethanol challenge showed greater allopregnanolone-induced impairments in the water maze compared to those receiving saline. The current study thus demonstrates that aged rats are especially vulnerable to anxiety-like behavior during ethanol withdrawal, and that repeated ethanol intoxications and withdrawals impair cognition in both age groups. Research targeting mechanisms and treatment of withdrawal-induced anxiety in the elderly is a promising avenue of future investigation.

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by

Adelle Novier, B.S., M.A.

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Approved by the Department of Psychology and Neuroscience

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Charles A. Weaver III, Ph.D., Chairperson

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Approved by the Dissertation Committee

_____

Jaime L. Diaz-Granados, Ph.D., Co-Chairperson

_____

Douglas B. Matthews, Ph.D., Co-Chairperson

_____

Joaquin N. Lugo, Jr., Ph.D.

_____

Sara L. Dolan, Ph.D.

_____

Eric L. Robinson, Ph.D.

Accepted by the Graduate School

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J. Larry Lyon, Ph.D., Dean

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ACTH	Adrenocorticotropic hormone
ARR	Aerial righting reflex
BEC	Blood ethanol concentration
CIE	Chronic intermittent ethanol
CRF	Corticotropin-releasing factor
HPA	Hypothalamic-pituitary-adrenal
MWM	Morris water maze
RR	Accelerating rotarod

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CHAPTER ONE

Introduction

Overview

Alcohol dependence and withdrawal among the aged is likely to become an important public health concern as the number of elderly alcohol consumers increases worldwide (Blazer & Wu, 2009; Geels et al., 2013; Wang & Andrade, 2013). It is estimated that individuals aged 65 years and older will account for 14 percent of the world's population by the year 2040 (Kinsella & He, 2009). Further, approximately 13 percent of elderly men and eight percent of elderly women reportedly engage in at-risk alcohol use (Blazer & Wu, 2009). Although the clinical and neurological symptoms of alcohol withdrawal have been well catalogued in adults, potential age-related differences in alcohol withdrawal in the elderly remain under-examined. Research suggests that the elderly may be especially vulnerable to the effects of acute alcohol (Thomas & Rockwood, 2001; Vestal et al., 1977). Furthermore, the elderly are particularly susceptible to alcohol-related physical and neural injury resulting from chronic consumption (Bridevaux et al., 2004; Menninger, 2002; Saunders et al., 1991). Elderly alcoholics may also experience more severe symptoms of alcohol withdrawal, and require increased medication and longer treatment duration, compared to younger individuals (Brower et al., 1994; Kraemer, Mayo-Smith, & Calkins, 1997; Liskow et al., 1989). However, examinations of ethanol withdrawal syndrome in aged rats are limited and inconsistent. The present study will thus add to the growing body of evidence investigating ethanol withdrawal in adult and aged rats following a model of repeated

ethanol intoxications and withdrawals. Furthermore, we will examine potential persistent deficits in motor and cognitive function resulting from chronic ethanol consumption, and investigate a potential mechanism underlying the increased sensitivity to ethanol-induced cognitive deficits observed in the aged.

Alcohol Withdrawal and Withdrawal-Induced Anxiety

Chronic, excessive consumption of alcohol has been shown to produce compensatory neurological changes that are associated with alcohol dependency. When alcohol consumption is reduced or ceased, symptoms of alcohol withdrawal emerge as the central nervous system seeks to reestablish homeostasis. As posited by Becker (2000), the clinical characteristics of alcohol withdrawal are generally divided into three categories: 1) hyperactivity of the autonomic system, 2) hyperexcitability of the central nervous system, and 3) distortion in sensation and perception. Over-excitation can manifest in physical symptoms, such as increased blood pressure and heart rate, sweating, headache, insomnia, and convulsions, as well as psychological symptoms of irritability, anxiety, and depression (De Witte et al., 2003; Hall & Zador, 1997; Myrick & Anton, 1998). The clinical and neurological signs of alcohol withdrawal have been well-studied in adult humans and rodents, but few investigations have focused on differences in the age-related manifestation of alcohol withdrawal in the elderly. As between one to three percent of the elderly are afflicted with alcohol use disorders (Caputo et al., 2012), differences in the symptoms and manifestations of alcohol withdrawal in the aged is likely to become an important area of investigation.

The symptoms and progression of alcohol withdrawal have been well-studied in adults. Depending upon the amount and duration of alcohol consumption, the severity of

alcohol withdrawal syndrome can range from moderate to life-threatening (Bayard et al., 2004). Three to six hours after alcohol cessation, while measureable amounts of blood alcohol are still present, initial symptoms of anxiety, insomnia, and tremors occur (Finn & Crabbe, 1997). Other symptoms of alcohol withdrawal appear between six and 48 hours later and include headache, irritability, nausea, disorientation, and sensitivity to light and sound. More serious symptoms, such as convulsions, hallucinations, and delirium tremens (DT's) may occur several days after the termination of alcohol consumption (Myrick & Anton, 1998) (for review, see Hall & Zador, 1997). The most serious symptom of alcohol withdrawal, the DTs, occurs in approximately five percent of patients and is characterized by increased heart rate, respiration, blood pressure, severe tremors, and hallucinations (Myrick & Anton, 1998). Although the mortality rate associated with the DTs was once between 15 and 25 percent (Victor, 1966), the current mortality rate is estimated to be approximately one percent with modern medical advancements (for review, see Schuckit et al. 1995). Symptoms of delirium typically abate by 72 hours post-consumption (Salum, 1975) but have been reported to persist for more than eight days (Stendig-Lindberg & Rudy, 1980). Animal models of alcohol dependency also undergo characteristic responses of withdrawal; signs of hyperexcitabilty in the rat include tremors of the limbs, body, or tail, rigidity, hyperreflexia, and spontaneous convulsions (Majchrowicz, 1981). Ethanol withdrawal has also been shown to result in changes in thigmotaxis (Slawecki & Roth, 2004), locomotion (Capaz et al., 1981; Devaud, Bartoo, & Malthankar, 2002; Erden et al., 1999) and rearing frequencies (Capaz et al., 1981) in the open field. In the rat, ethanol withdrawal typically peaks between eight and 24 hours post-ethanol, although the time-

course of withdrawal varies as a function of alcohol exposure method (Macey et al., 1996). Similarly, the severity of withdrawal differs depending upon the level of intoxication and length of ethanol exposure (Chung et al., 2008; Goldstein, 1972).

Many of the neurological and physiological symptoms of alcohol withdrawal occur as a result of neuroadaptations with GABAergic and glutamatergic systems (Davis & Wu, 2001; Hall & Zador, 1997). In particular, $GABA_A$ receptors have been implicated in many of the acute and long-term actions of alcohol (Kumar et al., 2009). Chronic alcohol produces alterations in receptor expression and function of $GABA_A$ receptors that may result in hyperexcitability of the central nervous system. For instance, $GABA_A$ peptide levels for subunits α1, α2, and α3 are down-regulated following long-term exposure to alcohol, while α4, β1, β2, β3, γ1, and γ2 expression is increased in the cerebral cortex (Kumar et al., 2009). The hippocampus similarly shows morphological changes and $GABA_A$-α4 peptide expression levels increase in response to chronic ethanol following 40 days of ethanol consumption (Matthews et al., 1998). Changes in $GABA_A$ receptor expression have also been observed in the cerebellum. Morrow et al. (1992) determined that chronic ethanol administration reduced subunit α1 mRNA while increasing α6 mRNA expression. Thus, alterations of $GABA_A$ receptors have been implicated in the manifestation of alcohol withdrawal syndrome and may contribute to symptoms such as anxiety and seizures (Littleton, 1998). Accordingly, the administration of selective GABA agonists or GABAmimetic drugs reduces the severity of alcohol withdrawal whereas GABA antagonists can exacerbate symptoms of withdrawal (Fadda et al., 1985; Frye, McCown, & Breese, 1983; Goldstein, 1973; Liljequist & Engel, 1982).

NMDA receptors have also been implicated in the manifestation of alcohol withdrawal syndrome. Long-term ethanol consumption results in up-regulation of glutamate receptors that likely contributes to hyperexcitabilty of the central nervous system (Davidson, Shanley, & Wilce, 1995; De Witte, 2004). Accordingly, ethanol withdrawal is associated with increased extracellular glutamate in the hippocampus (Dahchour & Witte, 1999) and rat striatum (Rossetti & Carboni, 1995). Hyperactivation of NMDA receptors is of particular concern as increased intracellular calcium levels can result in excitotoxicity and cell death in many areas of the brain; chronic alcoholism has been found to be associated with NMDA-induced neuronal loss in the cortex (Ahern, Lustig, & Greenberg, 1994; Chandler et al., 1993), hippocampus (Smothers, Mrotek, & Lovinger, 1997), and cerebellum (Iorio, Tabakoff, & Hoffman, 1993). NMDA is further implicated in the manifestation of ethanol withdrawal as NMDA receptor antagonists reduce seizure susceptibility and diminish the severity of withdrawal (Grant et al., 1990; Morrisett et al., 1990).

The intensity of ethanol withdrawal symptoms appears to be age-dependent. Chung et al. (2008) treated juvenile, adolescent, and young adult Long-Evans rats with liquid ethanol diet for two, three, or five weeks. The results revealed that juvenile rats were most adversely affected by termination of ethanol; adolescents displayed moderate withdrawal symptoms whereas adults were minimally affected. Additionally, limited research with older rodents suggests that aged rats have greater severity of ethanol withdrawal compared to young adults. Wood et al. (1982) treated three groups of mice (three, 14, and 25 months) with liquid ethanol diet for 14 days and rated ethanol intoxication and severity of withdrawal using a standardized scale. Older rodents were

significantly more intoxicated and exhibited greater withdrawal symptoms than did younger animals. Interestingly, differences in intoxication and withdrawal were not due to age-related differences in blood ethanol concentrations (BECs) (Wood et al., 1982). In contrast, Riihioja et al. (1999) used a chronic intermittent ethanol paradigm to investigate ethanol withdrawal in adult (3-4 months) and aged (29-30 month) male Wistar rats and found no age-related differences in severity of withdrawal. Animals underwent seven cycles of four-day ethanol administered by intragastric intubation followed by a three-day ethanol-withdrawal period. Notably, rather than administering equivalent quantities of ethanol, dosages were adjusted individually to control for age-related differences in intoxication level. Using this method, both adult and aged animals were able to achieve comparable intoxication and BEC levels despite old animals receiving significantly less alcohol per body weight. As previously reported (Wood et al., 1982), level of intoxication was found to be correlated with severity of withdrawal symptoms. Importantly, however, no age-related differences in severity of withdrawal were observed after controlling for intoxication (Riihioja et al., 1999). Given that few studies have examined the severity of ethanol withdrawal in aged rodents and results have been conflicting, future work assessing ethanol withdrawal in the aged is necessary.

Among the symptoms of ethanol withdrawal, anxiety is particularly problematic as it has been shown to be predictive of alcohol craving and relapse in humans (Roelofs, 1985; Willinger et al., 2002). Further, patients with comorbid diagnoses of alcohol dependency and anxiety disorder have more severe symptoms of alcohol withdrawal than those without an anxiety diagnosis (Johnston et al., 1991). Rodent models of ethanol withdrawal also exhibit increased anxiety-like behaviors following cessation of ethanol

(for review, see Gatch & Lal, 2001) that have been shown to persist for as long as four months after withdrawal (Santucci et al., 2008). In rodents, anxiolytic and anxiogenic behaviors have conventionally been examined using the elevated plus maze and open field tests (for review, see Kliethermes, 2005). Chronic ethanol treatment has consistently been shown to result in increased anxiety-like behavior in rats as evaluated by the number of open-arm entries and time spent in the open arms of the maze (File, Andrews, & Al-Farhan, 1993; Jung et al., 2000; Lal, Prather, & Rezazadeh, 1991; Moy et al., 1997; Rasmussen et al., 2001; Valdez et al., 2002). Anxiety-like behavior has also been observed in the open field as determined by percent time spent in the center area of the apparatus (Kumar et al., 2013). Additionally, the open field can also be used to observe hyperactivity in rodents undergoing withdrawal (Capaz et al., 1981; Erden et al., 1999; Mehta & Ticku, 1993; Waller et al., 1982). In contrast, few studies have reported hypoactivity in the open field during ethanol withdrawal (Maier & Pohorecky, 1989; Slawecki & Roth, 2004). Inconsistent findings from the open field have been attributed to differing rodent strains, age, lighting conditions, and other environmental and genetic influences. However, a review of the literature reveals that signs of hyperactivity have been observed more frequently in withdrawn rats than have signs of decreased locomotion (for review, see Kliethermes, 2005).

Although it is likely that anxiety and locomotor behavior associated with ethanol withdrawal may manifest differentially by age, no investigation has examined anxiety-like behavior specifically in aged rodents. One study of interest assessed the effects of ethanol withdrawal on open field performance in three month and older six-and-a-half month rats (Maier & Pohorecky, 1989). Animals were treated with ethanol via

intragastric catheter three times per day (8-11 g/kg ethanol) for two weeks and older animals were more vulnerable to withdrawal-induced hypoactivity than younger rats. Notably, six-and-a-half month rats exhibited decreased crossing and rearing in the maze compared to both same-aged controls and younger adults treated with ethanol (Maier & Pohorecky, 1989). These findings therefore suggest that withdrawal-induced anxiety-like behavior may be more pronounced in the aged. Future examination of the role of aging and anxiety during ethanol withdrawal may reveal important areas of interest for the development of novel therapeutic treatments for dependence in the elderly.

There is evidence that the severity of alcohol withdrawal symptoms may become exacerbated with repeated cycles of ethanol intoxication and withdrawal, a phenomenon known as "kindling" (Ballenger & Post, 1978; Becker, 1998). It is suggested that hyperexcitability of the central nervous system from chronic alcohol causes neuronal sensitization such that initially mild symptoms of alcohol withdrawal become progressively more severe with repeated occurrences (Becker, Diaz-Granados, & Weathersby, 1997). Indeed, patients exhibiting severe alcohol withdrawal symptoms and seizures are more likely to have a history of numerous prior detoxifications (Booth & Blow, 1993; Brown et al., 1988; Lechtenberg & Worner, 1990). Animal studies employing models of repeated ethanol withdrawal also provide support for the kindling mechanism. In rodents, the severity and duration of withdrawal-induced convulsions and seizures increases proportionately with the number of intoxication and withdrawal episodes (Becker, 1994; Becker et al., 1997; Clemmesen & Hemmingsen, 1984). Furthermore, the severity of ethanol withdrawal is greater in animals with multiple withdrawal periods compared to animals with an equivalent ethanol exposure time but

only one withdrawal episode (Becker et al., 1997; Becker & Hale, 1993). Rodents with a history of multiple withdrawal periods also exhibit decreased seizure threshold in response to seizure-inducing drugs (Becker et al., 1997; Kokka et al., 1993; McCown & Breese, 1993). Additionally, anxiety-like behavior has been shown to become increasingly sensitized with repeated ethanol withdrawal periods as assessed by the elevated plus maze (Hölter et al., 1998), open field (Maier & Pohorecky, 1989), and other anxiety tasks (Overstreet, Knapp, & Breese, 2002). As patients with a history of previous detoxifications have an increased risk of seizures and other severe withdrawal symptoms (Booth & Blow, 1993; Brown et al., 1988), the cumulative effects of multiple ethanol exposure and withdrawal episodes can have important implications for human alcoholics.

Aging and the Long-Term Effects of Chronic Ethanol Consumption

Considerable research documents physical and neuropsychological problems arising from long-term alcohol use in younger and older adults (Stevenson, 2005). Chronic alcoholism is associated with gray and white matter volume loss, corresponding increases in brain sulci and ventricle size, and increased cerebrospinal fluid volume (Pfefferbaum et al., 1992; Pfefferbaum et al., 1988; Pfefferbaum et al., 1995). Furthermore, chronic, heavy alcohol consumption causes atrophy of the cortex and hippocampus (Beresford et al., 2006; Kril et al., 1997; Pfefferbaum et al., 1992) which may negatively impact cognitive functioning. Accordingly, long-term alcohol use is associated with deficits in working memory, problem solving, visuospatial perception, and executive functioning skills (Ambrose, Bowden, & Whelan, 2001; Beatty et al., 1996; Beatty et al., 1993; Errico, Parsons, & King, 1991; Pitel et al., 2007). Importantly, although many cognitive impairments resulting from alcoholism are ameliorated with

abstinence, some aspects of performance such as spatial processing and long-term memory deficits may persist for years (Brandt et al., 1983; Fein et al., 2006; Mann et al., 1999).

The detrimental effects of chronic ethanol treatment on cognitive performance have also been well-studied in rodents. Some investigations have observed cognitive impairments in spatial tasks including the complex elevated maze (Franke et al., 1997), radial-arm maze (Arendt et al., 1989), and Morris water maze (Baydas, Yasar, & Tuzcu, 2005; Lukoyanov, Madeira, & Paula–Barbosa, 1999) in adult rats chronically treated with ethanol. In contrast, spatial memory was not affected by chronic ethanol treatment in other investigations (Blokland, Prickaerts, & Raaijmakers, 1993; Boulouard et al., 2002; Gál & Bárdos, 1994; Maier & Pohorecky, 1987). It is likely that method and length of ethanol exposure, strain of rat, and/or task complexity may influence spatial performance and account for the inconsistency in findings (Blokland et al., 1993). Notably, Maier & Pohorecky (1987) employed a repeated intoxication and withdrawal treatment paradigm whereupon rats were treated with ethanol via intragastric intubation for four, two week periods each separated by a two-week withdrawal. Neither repeated withdrawal nor eight consecutive weeks of ethanol exposure resulted in spatial learning impairments in the radial arm maze. The authors speculated that the maze may not have been a sensitive enough measure to observe cognitive impairments (Maier & Pohorecky, 1987). Other studies have reported chronic ethanol-induced deficits using more cognitively demanding tasks. For instance, Santucci et al. (2004) observed impairments in spatial working memory, but not reference memory, in the water maze following 26 days of continuous liquid ethanol diet. Additionally, Santin et al. (2002) treated animals with up to 20

percent liquid ethanol for approximately six months and found no effect of treatment on the initial acquisition of place learning. However, ethanol-exposed animals showed increased latency to the platform following a reversal task in which the platform was relocated 180 degrees from the original location. Thus, chronic ethanol treatment appears to influence behavioral flexibility and increase perseverative behavior (Santín et al., 2000), a finding which has been corroborated using other spatial performance tasks (Blokland et al., 1993; Gál & Bárdos, 1994).

Long-term alcohol consumption has also been shown to cause morphological deficits in the cerebellum which may negatively affect motor skills. Indeed, human alcoholics suffer atrophy of both the cerebellar hemispheres and the superior vermis (Sullivan et al., 2000a; Torvik & Torp, 1986) and show associated deficits in gait, balance, and coordinated movement even during abstinence (Sullivan et al., 2000b; York & Biederman, 1991). Importantly, although the cerebellum shows normal aged-related declines in volume, gray matter loss in the cerebellar hemispheres is exacerbated in elderly alcoholics compared to non-abusers (Sullivan et al., 2000a). Similarly, rodents treated with chronic ethanol also show cerebellar alterations. Rats consuming ethanol for twelve consecutive months showed reduced dendritic branch density and length, as well as decreased spine density, on cerebellar Purkinje cells (Tavares, Paula-Barbosa, & Gray, 1983). Furthermore, as few as three months of chronic ethanol consumption can decrease the spontaneous firing of Purkinje cells and result in significant motor impairments in tasks such as the rotarod and runway in mice (Servais et al., 2005). Therefore, studies with both humans and rodents suggest that long-term alcohol consumption may result in brain damage that negatively affects both cognitive and motor functioning. Importantly,

the aging population may be particularly vulnerable to the health effects associated with chronic alcohol use.

Limited work suggests that the elderly are uniquely sensitive to the short-term and chronic effects of alcohol consumption compared to younger adults. For instance, changes in body composition with age may decrease total body water volume and result in higher circulating blood alcohol levels in response to any given acute dose (Jones & Neri, 1985; Menninger, 2002; Vestal et al., 1977) (but see Novier et al., 2013). Studies with rodents have revealed that ethanol metabolism also decreases with age as hepatic blood flow is reduced and liver enzyme activity declines (Hahn & Burch, 1983; Kim, Kim, & Sohn, 2003; Menninger, 2002; Ritzmann & Springer, 1980; Seitz et al., 1992). Furthermore, the elderly are particularly susceptible to the health-related problems associated with alcoholism including falls and bone fractures, dementia, and organ damage (for review, see Menninger, 2002). Research with humans suggest that alcoholism interacts negatively with the natural aging process making this population of drinkers especially vulnerable to the deleterious effects of ethanol (Stevenson, 2005). Indeed, long-term alcohol consumption has been shown to accelerate hippocampal and cerebellar atrophy (Sullivan et al., 2000a; Sullivan et al., 1995) and to result in global reductions of white and gray matter volume (Pfefferbaum et al., 1992) that is attributed to the combination of both alcohol use and aging.

Importantly, aged rodents also appear to be especially sensitive to the behavioral effects of ethanol. Indeed, older rodents show greater sedation (Abel & York, 1979; Ott, Hunter, & Walker, 1985; Ritzmann & Springer, 1980) (but see Work, 1983), hypothermia (Wood & Armbrecht, 1982), ataxia (Novier et al., 2013; Van Skike et al.,

2010), and cognitive impairments (Novier et al., 2013) following acute ethanol than younger animals. In response to chronic ethanol, aged mice experience enhanced signs of intoxication and exhibit severe ethanol withdrawal symptoms. Notably, these age differences were apparent after only 14 days of liquid diet (Wood et al., 1982). While recent work in our laboratory has examined age-related effects of acute ethanol on cognitive and motor performance, few studies have examined persistent deficits following long-term ethanol exposure in aged animals. Blokland et al. (1993) treated three- and 18-month Wistar rats with 20 percent liquid ethanol diet for six months and assessed spatial performance using both the Morris water maze and cone-field task. Interestingly, although aged rats did show impaired latency to the platform relative to adults, chronic ethanol treatment had no influence on spatial learning in either age group. In contrast, both three- and 19-month rats showed spatial acquisition deficits in the water maze following 45 days of 2.0 g/kg ethanol when administered via intraperitoneal (i.p.) injection (Baydas et al., 2005). The current investigation will thus add to the limited research examining the interaction between aging, chronic ethanol, and cognitive performance.

Allopregnanolone and Ethanol-Induced Cognitive Impairment

Allopregnanolone (3α-hydroxy-5α-pregnan-20-one or 3α,5α-THP) is a potent positive allosteric modulator of $GABA_A$ receptors that mediates many of the behavioral and neural actions of ethanol (Khisti et al., 2003; Morrow et al., 1999; VanDoren et al., 2000). Allopregnanolone is a progesterone metabolite synthesized in the brain via the enzymes 5α-reductase, which reduces progesterone to 5α-dihydroprogesterone (5α-DHP), and 3α-hydroxysteroid oxidoreductase (3α-HSOR), which converts 5α-DHP into

allopregnanolone (Cheney et al., 1995; Dong et al., 2001; Morrow et al., 1999). Similar to the mechanism of actions of barbiturates, allopregnanolone enhances the inhibitory effects of the $GABA_A$ receptor by potentiating GABA-activated chloride flux in the central nervous system (Majewska et al., 1986). As a $GABA_A$ modulator, allopregnanolone administration induces anxiolysis (Bitran, Hilvers, & Kellogg, 1991; Brot et al., 1997), sedation (Bitran et al., 1991; Khisti et al., 2003), cognitive impairments (Chin et al., 2011; Matthews et al., 2002), anti-convulsant (Mareš et al., 2006) and analgesic (Korneyev & Costa, 1996) properties. Allopregnanolone plays a vital role in hormone fluctuations during pregnancy (Concas et al., 1998) and has been recently implicated in reproductive mood disorders (Bäckström et al., 2014; Schiller, Schmidt, & Rubinow, 2014). Further, allopregnanolone modulates hypothalamic-pituitary-adrenal (HPA) axis activation by stimulating the adrenals to release progesterone and allopregnanolone into circulation in response to stress (Barbaccia et al., 1996; Biggio et al., 2014; Morrow et al., 1999; Purdy et al., 1991). Thus, HPA axis activation acts as a mechanism to rapidly elevate brain and plasma allopregnanolone levels as a possible feedback mechanism for regulating the stress response (Morrow et al., 1999).

Remarkably, the behavioral effects of allopregnanolone are strikingly similar to those seen in response to acute ethanol. Specifically, ethanol also has anti-convulsant, sedative-hypnotic, anxiolytic, and cognitive-impairing properties (for a review, see Kumar et al., 2009). Due to the similarity in the behavioral effects of ethanol and allopregnanolone, neurosteroids including allopregnanolone have been posited to mediate some of ethanol's actions at $GABA_A$ receptors (Grobin et al., 1998). Indeed, acute ethanol administration elevates plasma and cerebral cortical allopregnanolone levels in a

dose- and time-dependent manner in both male and female rats (Morrow et al., 1999; VanDoren et al., 2000) (for review, see Silvers et al., 2003). Ethanol acts as a physiological stressor that activates the HPA axis, increasing corticotropin-releasing hormone (CRF) in the hypothalamus and stimulating release of adrenocorticotropic hormone (ACTH) from the anterior pituitary and into the plasma. Once ACTH reaches the adrenals, neuroactive steroids including progesterone and allopregnanolone are released into general circulation (Morrow et al., 2004). Ethanol appears to increase allopregnanolone levels in part by modulating neurosteroid biosynthesis. Pre-administration of the 5α-reductase inhibitor, finasteride, has been shown to partially attenuate ethanol-induced allopregnanolone release as observed in the cerebral cortex of male rats (Morrow et al., 1999). Ethanol-induced allopregnanolone release is biphasic; doses between 2.0 and 3.0 g/kg ethanol result in the greatest increase of 3α,5α-THP, as much as a 700 percent increase in the cortex of male rats relative to saline controls, whereas higher doses of ethanol have a diminished effect. Additionally, allopregnanolone peaks between 40 and 80 minutes post-injection. Thus, 3α,5α-THP likely contributes to some of the behavioral effects of acute ethanol observed during this time period (Morrow et al., 1999).

Allopregnanolone has been implicated in many of the behavioral effects of ethanol. For instance, VanDoren et al. (2000) investigated the relationship between 3α,5α-THP levels and ethanol-induced sedation. As previously shown, systemic administration of ethanol increased cerebral cortical levels of allopregnanolone in a dose-dependent manner. Importantly, a significant correlation was found between cerebral cortical 3α,5α-THP levels and ethanol-induced sleep time, suggesting that

allopregnanolone likely contributes to the sedative/hypnotic effects of ethanol via $GABA_A$ receptor activation (VanDoren et al., 2000). Allopregnanolone is further implicated in the sedative/hypnotic effects of ethanol as adrenalectomy reduces ethanol-induced increases of cortical and plasma 3α,5α-THP and decreases the duration of loss of righting reflex (Khisti et al., 2003). Notably, allopregnanolone does not modulate ethanol-induced ataxia as neither finasteride pretreatment nor adrenalectomy influences motor coordination in rats (Khisti et al., 2004) Furthermore, allopregnanolone also appears to be involved in the anti-convulsant effects of ethanol as pre-treatment with finasteride partially reverses ethanol-induced increases in seizure threshold (VanDoren et al., 2000). Additionally, allopregnanolone may play a role in the rewarding and reinforcing effects of ethanol. Barbaccia et al. (1999) examined allopregnanolone levels in the cortex and hippocampus of both ethanol-preferring and non-preferring Sardinian rats and found, as expected, that acute ethanol increased 3α,5α-THP levels. However, allopregnanolone levels were significantly more elevated in ethanol-preferring animals compared to non-preferring (Barbaccia et al., 1999). Similarly, allopregnanolone administration dose-dependently increases ethanol-reinforced operant responding, further suggesting that neurosteroids including allopregnanolone mediate ethanol reinforcement in rodents (Janak, Redfern, & Samson, 1998). Finally, an abundance of research has investigated the possible role of allopregnanolone in ethanol's cognitive-impairing effects.

Acute administration of allopregnanolone has been shown to influence the function and neurophysiology of the hippocampus, a structure profoundly sensitive to the effects of ethanol (for review, see White et al., 2000). For example, 17.0 mg/kg and 20.0

mg/kg acute allopregnanolone impair spatial memory in the hippocampal-dependent Morris water maze task in a dose-dependent manner (Matthews et al., 2002). Furthermore, both allopregnanolone and ethanol administration *in vivo* results in inhibition of spontaneous firing in hippocampal pyramidal cells, and ethanol-induced hippocampal inhibition is blocked by pretreatment of finasteride (Tokunaga et al., 2003). Similarly, finasteride pretreatment also reverses ethanol-induced inhibition of spontaneous firing rates in the medial septum/ diagonal band of Broca (VanDoren et al., 2000). Thus, elevated allopregnanolone levels appear to contribute to the cognitive-impairing effects of acute ethanol (for review, see Silvers et al., 2003). Interestingly, allopregnanolone does not appear to produce differential spatial memory impairments between adolescent and adult rats. Chin et al. (2011) trained adolescent (PD 28-30) and adult (PD 70—72) male Sprague-Dawley rats on a standard version of the water maze for six days. Animals were then administered 9.0 or 18.0 mg/kg allopregnanolone or a vehicle control before spatial memory testing. Although 18.0 mg/kg allopregnanolone increased latency and path length to the hidden platform in both age groups, no difference between the performance of adolescent and adult rats was found (Chin et al., 2011).

Given the projected increase in the number of elderly alcohol consumers (Blazer & Wu, 2009; Caputo et al., 2012; Johnson, 2000), the role of allopregnanolone in alcohol-induced cognitive impairments is an important but unexamined area of investigation. Limited evidence suggests that aged rodents are uniquely sensitive to the effects of acute ethanol on cognitive memory. Novier et al. (2013) reported that aged rats were more impaired in the water maze than younger animals following acute ethanol and these deficits were most apparent at the higher 1.5 /kg and 2.0 g/kg ethanol doses. There

is some indication that elderly rodents may also be more sensitive to neuroactive steroid release compared to younger animals. Barbaccia et al. (1998) found that brain cortical and plasma allopregnanolone levels were significantly greater in aged rats compared to adults following an acute stress paradigm. While it has not been directly investigated, we broadly theorize that aged rats might also show greater increases of allopregnanolone in response to acute ethanol. Therefore, potential age-related increases in allopregnanolone release may provide a potential mechanism for the exacerbation of ethanol-induced cognitive impairments in the aged.

Allopregnanolone also appears to contribute to the maintenance of ethanol tolerance, dependence, and withdrawal as examined in rodents (for review, see Morrow et al., 2001). While acute ethanol results in elevated brain allopregnanolone levels, (Morrow et al., 1999; VanDoren et al., 2000), male rats made dependent on ethanol via liquid ethanol diet actually show decreased 3α,5α-THP levels in the cerebral cortex (Janis et al., 1998). Notably, allopregnanolone levels returned to baseline during withdrawal, six to eight hours after ethanol removal (Janis et al., 1998). Similarly, chronic intermittent ethanol administration decreases 3α,5α-THP levels (as well as its precursor, 5α-reductase) in the hippocampus of adult rats (Cagetti et al., 2004). Moreover, dependent and withdrawn rodents show decreased elevations of allopregnanolone when challenged with an acute dose of ethanol compared to controls (Morrow et al., 2001). Therefore, tolerance to the behavioral and physiological effects of ethanol may involve changes in ethanol-induced allopregnanolone release (Morrow et al., 2001). Accordingly, allopregnanolone also appears to play a role in tolerance to the memory-impairing effects of ethanol. Adolescent rats treated with chronic intermittent ethanol (CIE) were less

adversely affected by acute ethanol in the water maze and displayed a reduction in ethanol-induced increases of allopregnanolone in the hippocampus compared to chronic controls (Silvers et al., 2006). Additionally, allopregnanolone is further implicated in the manifestation of ethanol withdrawal as allopregnanolone administration reduces withdrawal severity. Finn et al. (1995) reported that Withdrawal Seizure-Prone mice experienced significantly fewer handling-induced convulsions when treated with 3α,5α-THP during withdrawal. Similarly, administration of allopregnanolone protects against bicuculline-induced seizure threshold in withdrawn rats (Devaud, Purdy, & Morrow, 1995a).

Although research with rodents strongly implicates allopregnanolone in the long-term effects of ethanol, investigations with human alcohol drinkers have produced conflicting results. Acute alcohol intoxication has been shown to increase serum allopregnanolone levels in male and female adolescents admitted to the emergency room (Torres & Ortega, 2003, 2004). However, low-dose alcohol failed to influence allopregnanolone levels in adult males (Porcu et al., 2010). Interestingly, allopregnanolone levels do appear to be altered in humans during alcohol withdrawal. Romeo et al. (1996) examined neuroactive steroid levels in nine alcoholic patients during the early stages of withdrawal and found significantly decreased plasma allopregnanolone compared to controls. Notably, decreased allopregnanolone levels were observed between four and five days after alcohol cessation when anxiety and depression scores were elevated (Romeo et al., 1996). As increased allopregnanolone levels are associated with anxiolytic and antidepressant properties in rodents (Hirani, Khisti, & Chopde, 2002;

VanDoren et al., 2000), fluctuations in allopregnanolone during withdrawal may contribute to alcohol withdrawal syndrome in humans (Romeo et al., 1996).

Primary Investigative Goal

The present experiments will add to limited research examining the differential effects of age on ethanol withdrawal and the behavioral deficits associated with chronic ethanol consumption. We will examine the severity of ethanol withdrawal and withdrawal-induced anxiety from liquid ethanol diet in adult and aged rats. Further, we will investigate persistent deficits in motor and cognitive function in both age groups. We theorize that aged rats will be more vulnerable to ethanol withdrawal and the long-term deficits caused by chronic consumption. Lastly, we will examine the effects of an acute dose of allopregnanolone on water maze performance in adult and aged rats. We hypothesize that aged animals will be more vulnerable to allopregnanolone-induced impairments compared to younger rats. The long-term effects of ethanol are likely to become especially important as the number of elderly alcohol drinkers increases. Research on alcohol's effects during senescence is vital to understanding the unique consequences of long-term alcohol consumption in the elderly population.

CHAPTER TWO

Material and Methods

Animals and Liquid Diet Administration

Thirty-two adult (PD70) and 38 aged (18 months) male Sprague-Dawley rats were purchased from Harlan Laboratories (Indianapolis, IN) and housed individually at Baylor University in an animal colony approved by the Institutional Animal Care and Use Committee. Rats consumed a nutritionally complete liquid diet (Lieber-DeCarli '82, Bio-Serv, New Jersey) with ethanol, as previously described (Devaud et al., 1995b; Matthews et al., 1998). Animals were allowed two days to acclimate to the liquid diet before 5.0 percent (v/v) ethanol was added to the diet for seven days. Ethanol concentration was increased to 7.5 percent (v/v) ethanol for the following seven days. Previous data has shown that this method produces average daily ethanol consumption levels of approximately 8-10 g/kg and mean blood ethanol concentrations of 223 ± 21 mg/dl (Morrow et al., 1992). Following the initial 14 days of ethanol exposure, a 24-hour withdrawal period was initiated by substituting ethanol with maltose dextrin to equate caloric intake. Animals underwent six additional cycles of seven days of ethanol exposure (7.5 percent) separated by one 24-hour alcohol-free period. Thus, animals consumed ethanol for eight weeks in total and underwent seven 24-hour ethanol withdrawal periods. A group of control animals was pair-fed an identical diet with maltose dextrin substituted for ethanol for the entire exposure period. For a timeline of ethanol treatment, see Figure 1. Water was available ad libitum throughout the study, and body weights and dietary consumption were monitored daily. Separate two-way (Week x

Age) repeated measures ANOVAs were conducted on the weekly averages of liquid ethanol diet and grams of ethanol per body weight consumed. Blood was collected by tail-nick one hour following the initiation of the dark cycle in the middle of Cycles 2, 5, and 7 for blood ethanol concentration (BEC) analysis via the Analox AM1 Analyser (Hammersmith, London) protocol.

Acclimate	Cycle 1 (5%)	Cycle 2 (7.5%)	WD 1	Cycle 3 (7.5%)	WD 2	Cycle 4 (7.5%)	WD 3
Cycle 5 (7.5%)	WD 4	Cycle 6 (7.5%)	WD 5	Cycle 7 (7.5%)	WD 6	Cycle 8 (7.5%)	WD 7

Figure 1. Timeline of ethanol treatment. WD denotes a 24-hour withdrawal period.

Ethanol Withdrawal

During each 24-hour ethanol-free period, observational withdrawal signs were assessed at 2, 4, 8, 12, and 24 hours post ethanol using a standardized behavioral rating scale (see Table 1 as reproduced from Hemmingsen et al., 2004). Rigidity, tremor, irritability, and hypoactivity have been shown to be valid signals of ethanol withdrawal in the rat (Clemmesen & Hemmingsen, 1984; Hemmingsen, Clemmesen, & Barry, 1984; Riihioja et al., 1999; Riihioja et al., 1997). The total score was defined as the sum of each of the scores from the four categories as agreed upon by two experimenters. Withdrawal scores were analyzed via separate two-way (Session x Hour) ANOVAs for adult and aged rats.

Table 1: Rating of Withdrawal Symptoms

Score	Observations
	RIGIDITY, based on the position of the tail, the gait and the stance
0	Not present
1	Only tail stiffness observable
2	Some extension and abduction of extremities, somewhat broad-based stance or gait
3	Rigid posture with maximally extended and abduced extremities
	INTENTIONAL TREMOR, tremulous ataxia occurring during movement or intended movement
0	Not present
1	Slight
2	Moderate
3	Severe
	IRRITABILITY, increased response to external stimuli (e.g., enhanced startle reflex, unusual aggressiveness on handing)
0	Not present
1	Slight
2	Moderate
3	Severe
	HYPOACTIVITY, decrease of the general level of locomotor activity
0	Normal or slightly increased activity
1	Slightly reduced level of activity
2	Clear hypoactivity
3	Almost complete hypoactivity

Behavioral Testing

Further behavioral testing was performed to examine the effects of chronic ethanol treatment (CE) compared with chronic control treatment (CC). Anxiety was assessed on the elevated plus maze immediately following the final 24-hour withdrawal assessment (WD 7). Forty-eight hours after cessation of ethanol, animals were assessed in the open field apparatus. Further cognitive and motor performance testing was undertaken 14 days following the removal of ethanol to avoid any residual effects of ethanol or withdrawal. During cessation, both ethanol and control animals received standard lab chow. After 14 days ethanol-free, animals underwent six days of training in the Morris water maze (MWM training). The following day, half of each group was randomly assigned to receive an acute dose of ethanol or saline and assessed for aerial righting reflex and spatial memory in the MWM (ethanol test). Thus, eight groups were formed: 1) adult CE + acute ethanol, 2) adult CE + acute saline, 3) adult CC + acute ethanol, 4) adult CC + acute saline, 5) aged CE + acute ethanol, 6) aged CE + acute saline, 7) aged CC + acute ethanol, and 8) aged CC + acute saline. Twenty-four hours following ethanol testing, the platform of the MWM was relocated 180 degrees for three days of reversal training to examine response perseveration (reversal paradigm). Twenty-four hours after the culmination of the reversal paradigm, all rats received an acute dose of allopregnanolone and were tested once again in the MWM. For a timeline of anxiety and behavioral testing, see Figure 2. During the course of the study, two aged CC + acute saline animals and two aged CE + acute ethanol animals died.

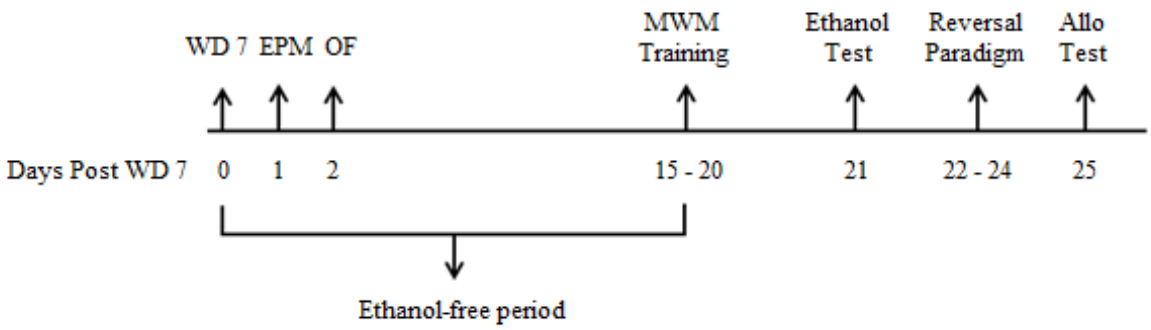


Figure 2. Timeline of behavioral testing. Anxiety and behavioral testing will be conducted after the final withdrawal assessment. Elevated plus maze denoted by EMP; open field test denoted by OF.

Elevated Plus Maze

Immediately following the final 24-hour withdrawal rating (WD 7), anxiety was measured on an elevated plus maze that was located in a behavioral room isolated from animal caging and housing. The apparatus was elevated 50 cm from the ground and consisted of four arms 50 cm in length and 11 cm wide. The closed arms were 40 cm in height and located at opposing sides of the maze. Animals were relocated to the testing room 30 minutes prior to experimentation and allowed to acclimate. For each trial, the animal was placed in the central location facing an open arm and allowed 5 minutes to explore the maze. The apparatus was wiped clean with a 70 percent ethanol solution between trials, and all trials were video recorded. The number of open and closed arm entries, as measured by entry of all four paws, and the time in seconds spent in the open and closed arms was recorded. Separate one-tailed independent-samples *t*-tests were conducted on the percent of time spent in the open arms of the maze and the number of arm entries for adult and aged rats.

Open Field

Anxiety and locomotion were measured by open field 48 hours after the final withdrawal day (WD 7). The apparatus was located in a behavioral room isolated from animal caging and housing, and measured 60.96 cm (24 inches) in height and 60.96 cm in diameter. Animals were placed in the center of the apparatus and allowed 5 minutes to explore the maze. The maze was wiped clean with a 70 percent ethanol solution between trials. Anxiety was assessed by percent of time spent exhibiting thigmotaxic behavior, defined as exploration of the outer 35 percent perimeter of the maze. Locomotion was evaluated by distance traveled in meters. Thigmotaxis and path length were recorded using the HVS Image 2100 Plus tracking system (Buckingham, UK). Additionally, instances of rearing, measured as having two paws off the ground, were documented. Distance, percent of time in thigmotaxis, and number of rears were analyzed via separate one-tailed independent-sample *t*-tests.

Morris Water Maze Training

Apparatus: The water maze (MWM) tank, which measured 125.7 cm in diameter and 59.7 cm in height, was located in an animal testing room surrounded by several extra maze cues. The water in the tank was made opaque with black nontoxic tempera paint and filled to a depth of 31 cm. The water was maintained at room temperature (~25°C). A Plexiglas platform measuring 30 cm tall and 12.5 cm in diameter was placed in the middle of the South-East quadrant, one cm below the water level.

Training: Animals were trained to locate a submerged platform in a fixed location of the MWM for six days after 14 days ethanol-free. Each day consisted of four trials in which the rat was released facing the wall from one of the four cardinal compass points

as determined by a Latin square design, as previously described (Chin et al., 2011). Each trial had a maximum latency of 45 seconds; if the rat did not locate the platform within 45 seconds, it was gently guided there. All rats were allowed to stay on the platform for 10 seconds after which they were removed and placed in a plastic holding cage for an inter-trial interval ranging between 30 and 60 seconds. Following the trials, rats were dried and returned to the home cage. Latency, path length, swim speed, and thigmotaxis were recorded using the HVS Image 2100 Plus tracking system (Buckingham, UK). Separate two-way (Chronic drug x Day) repeated measures ANOVAs were conducted on latency, path length, swim speed, and thigmotaxis data.

Ethanol Test

Aerial Righting Reflex (ARR)

Twenty-four hours after the last water maze training day, adult and aged rats were randomly assigned to receive an acute i.p. injection of 1.5 g/kg ethanol or saline during testing. Aerial righting reflex was assessed 15 minutes post-injection. A ruler taped vertically above a foam pad served as the test apparatus, as previously described (Novier et al., 2013). Rats were held in the supine position and released from a height of 5 inches (12.7 cm) above a foam pad. Successful righting required that three of four paws make direct contact with the foam pad on two of three releases. Success was agreed upon by two experimenters. If the animal failed to display successful righting, release height was increased by five inch increments until successful righting was achieved. Subjects were not released more than 25 inches (63.5 cm) above the foam pad. Animals who failed to achieve successful righting at a height of 25 inches were assigned a score of 30 for all

statistical analyses. Righting height was analyzed with a two-way (Chronic drug x Acute drug) ANOVA.

Water Maze Testing

Spatial memory was assessed 30 minutes post-injection of 1.5 g/kg ethanol or saline (and 15 minutes after ARR). Groups were formed and dosed as above (ARR). During testing, rats were given four trials starting from each cardinal direction and allowed 45 seconds to locate the hidden platform, which remained in the same spatial location as used during training. Immediately following the fourth testing trial, tail blood was collected for BEC assay via the Analox AM1 protocol. Latency, path length, swim speed, and thigmotaxis were analyzed independently via two-way (Chronic drug x Acute drug) ANOVAs.

MWM Reversal Paradigm

Twenty-four hours following the acute ethanol test day, the hidden platform in the MWM was relocated to the opposite quadrant to test for response perseveration and for enhanced detection of spatial impairments (Silvers et al., 2006). Animals were given three additional training days, four trials per day, as described above. Latency, path length, swim speed, and thigmotaxis were analyzed independently via three-way (Day x Chronic drug x Acute drug) ANOVAs.

Allopregnanolone Test

Twenty-four hours after the last day of reversal training, all animals received an acute injection of 15.0 mg/kg allopregnanolone (suspended in 20 percent 2-hydroxypropyl-b-cyclodextrin and saline after at least 80 minutes of sonification) and

underwent four additional testing trials in the MWM as above. The platform remained in the same spatial position as was used during reversal paradigm training. Performance in the water maze was assessed 20 minutes post i.p. injection, as previously described (Matthews et al., 2002). Water maze performance during the allopregnanolone test day was analyzed via independent two-way (Chronic drug x Prior acute drug) repeated measures ANOVAs for latency, path length, swim speed, and thigmotaxis.

CHAPTER THREE

Results

Body Weights

Body weights recorded during ethanol exposure indicate that pair-feeding was successful. A significant main effect of time revealed that body weights increased throughout treatment for both adults, $F(7, 210) = 256.30$, $p < 0.001$, and aged rats, $F(7, 245) = 34.68$, $p < 0.001$. However, no significant difference in body weight was found between animals exposed to chronic ethanol and chronic control diet for either adults, $F(1, 210) = 0.75$, $p > 0.05$ (Figure 3a), or aged, $F(1, 245) = 0.00$, $p > 0.05$ (Figure 3b).

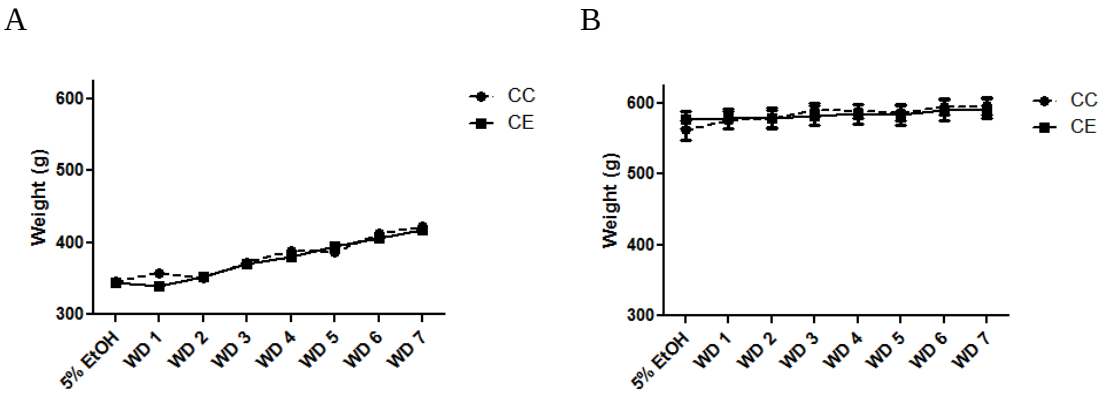


Figure 3. Body weights collected every eight days during ethanol treatment prior to withdrawal. (A) No difference was found between adults exposed to chronic control diet and those exposed to chronic ethanol diet. (B) No difference was found between aged animals exposed to chronic control diet and those exposed to chronic ethanol diet. Error bars denote SEM.

Ethanol Diet

A significant interaction between session and age revealed that the amount of diet consumed increased across withdrawal session and aged rats consumed more diet than

adult animals, $F(7, 231) = 24.65$, $p < 0.001$. Bonferroni post-tests indicated this difference was significant during Sessions 1 - 6 (Figure 4a). However, when accounting for body weight, aged animals consumed significantly less grams of ethanol as indicated by a significant Session x Age interaction for grams of ethanol per body weight, $F(7, 231) = 8.57$, $p < 0.001$. Bonferroni post-tests revealed these age differences to be significant for Sessions 1- 8 (Figure 4b).

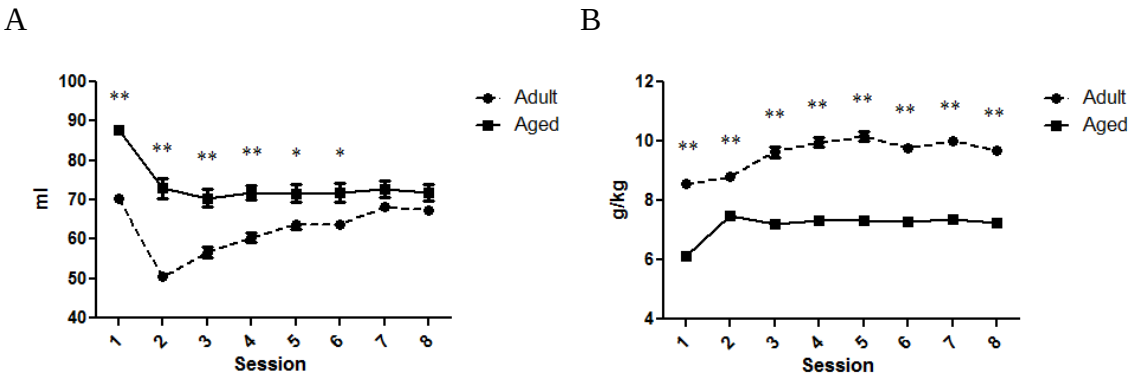


Figure 4. Average liquid diet and ethanol consumed per treatment week. (A) Aged animals consumed significantly more ml of ethanol diet compared to adults. (B) Adults consumed significantly more g/kg of ethanol compared to aged animals. * $p < 0.05$, ** $p < 0.01$, error bars denote SEM.

Blood Ethanol Concentration (BEC) Levels

A significant interaction between age and day was found for blood ethanol concentration (BEC) levels, $F(2, 66) = 6.27$, $p < 0.01$. Bonferroni post-tests indicated that adult animals had significantly higher BECs than aged animals during all tested time points (Figure 5).

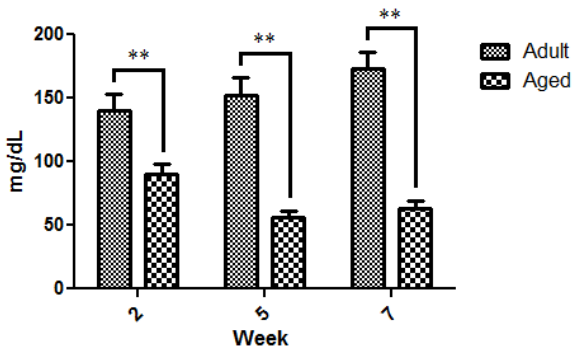


Figure 5. Blood ethanol concentration (BEC) levels as determined from blood collection in the middle of Weeks 2, 5, and 7. Adults had significantly higher BECs during all weeks compared to aged. ** $p < 0.01$, error bars denote SEM.

The significant difference in ethanol consumption and BECs between age groups suggests that adult and aged rats will have unique responses to ethanol withdrawal and subsequent behavioral testing. Due to these inherent differences in ethanol consumption and intoxication, adult and aged rats will be analyzed independently for all subsequent statistical analyses (Van Skike, 2013).

Ethanol Withdrawal

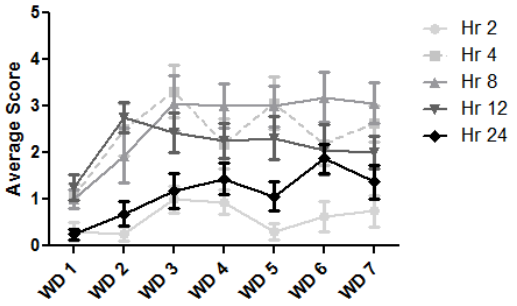
Adult: A significant interaction between session and hour was found for withdrawal scores for adult rats, $F(24, 450) = 1.59$, $p < 0.05$ (Figure 6a). Bonferroni post-tests were used to probe the significant interaction between session and hour and the significant comparisons are shown in Table 2. Average withdrawal scores by hour are shown in Figure 7a, and average withdrawal scores by session are shown in Figure 8a. Aged: A significant Session x Hour interaction was found for withdrawal scores for aged rats, $F(24, 540) = 1.74$, $p < 0.05$ (Figure 6b). The interaction was probed for significant comparisons as determined by Bonferroni post-tests and the results are shown in Table 2.

Table 2. Significant comparisons for withdrawal by hour and session; $p < 0.05$

Withdrawal Session	Adult	Aged
WD 1	NS	NS
WD 2	Hr 2 vs 4, 8, 12 Hr 24 vs 4, 12	NS
WD 3	Hr 2 vs 4, 8 Hr 24 vs 4, 8	NS
WD 4	Hr 8 vs 2, 24	Hr 12 vs 2, 4, 8, 24
WD 5	Hr 2 vs 4, 8, 12 Hr 24 vs 4, 8	Hr 8 vs 12
WD 6	Hr 2 vs 4 , 8	
WD 7	Hr 2 vs 4, 8 Hr 8 vs 24	

Further, withdrawal scores by hour for aged rats are shown in Figure 7b, and average withdrawal scores by session are shown in Figure 8b.

A



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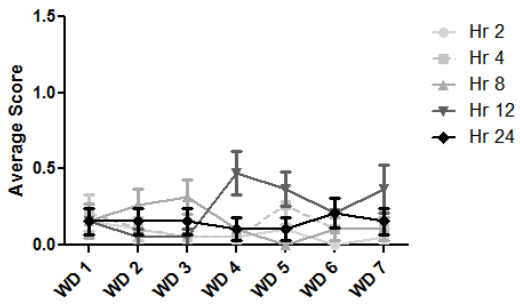

Figure 6. Withdrawal scores for adult and aged animals. (A) A significant interaction was found between session and hour for adults and (B) aged animals. $p < 0.01$, error bars denote SEM.

A B

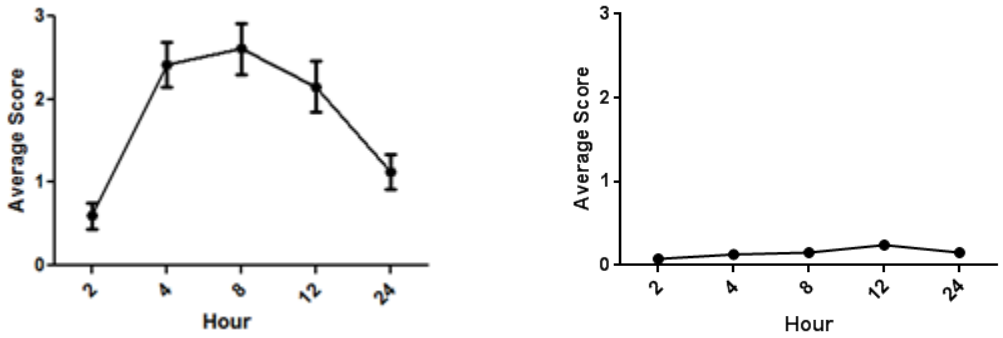

Figure 7. Withdrawal scores by hour for (A) adult and (B) aged animals. Error bars denote SEM.

A B

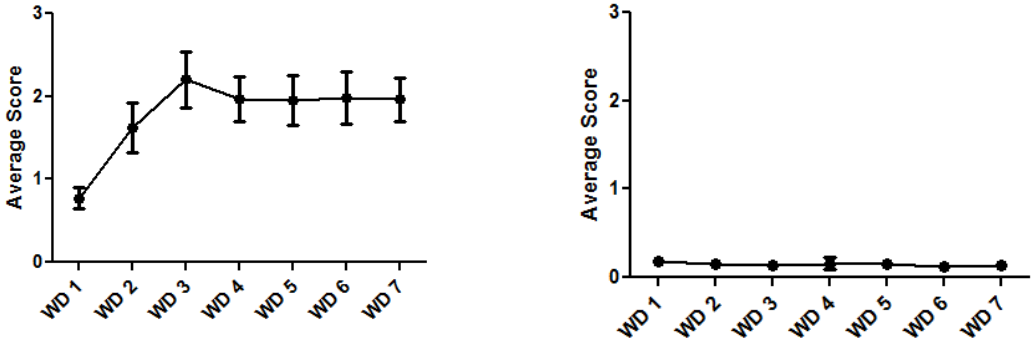

Figure 8. Withdrawal scores by session for (A) adult and (B) aged animals. Error bars denote SEM.

Elevated Plus Maze

Adult: The percentage of time spent in the open arms of the elevated plus maze did not significantly differ between adults receiving chronic ethanol and those receiving chronic control diet, $t(30) = 1.53$, $p > 0.05$ (Figure 9a). Similarly, chronic drug treatment did not influence the number of open arm entries, $t(30) = 1.28$, $p > 0.05$ (data not shown) or the number of closed arm entries, $t(30) = 0.49$, $p > 0.05$ (Figure 10a).

Aged: Aged rats treated with chronic control diet spent a greater percent of time in the open arms than those treated with chronic ethanol, $t(35) = 3.93$, $p < 0.001$ (Figure 9b). Similarly, ethanol-treated aged animals made significantly greater open arm entries than controls, $t(35) = 2.38$, $p < 0.05$ (data not shown). Additionally, aged animals treated with chronic ethanol made significantly more closed arm entries than did controls, $t(35) = 2.03$, $p < 0.05$ (Figure 10b).

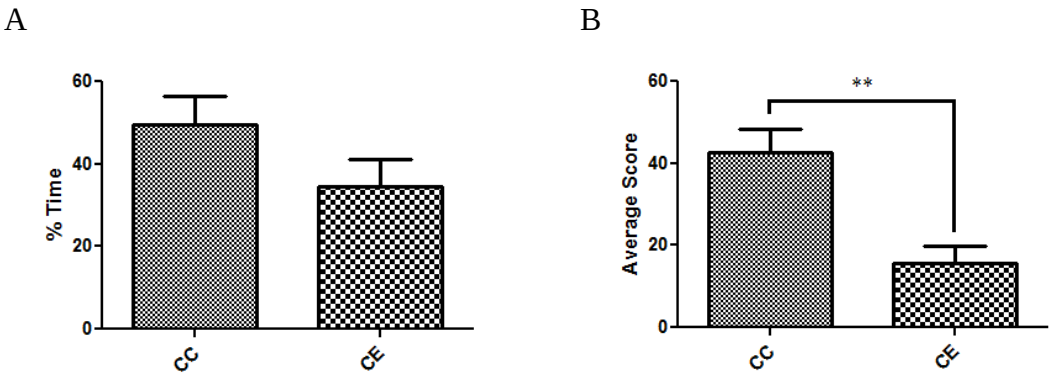

Figure 9: Percent of time spent in the open arms of the elevated plus maze (EPM) for chronic control (CC) and chronic ethanol (CE) groups. (A) Adult CE animals spent marginally less time in the open arms than CC. (B) Aged CE animals spent significantly less time in the open arms than CC. ** $p < 0.001$, error bars denote SEM.

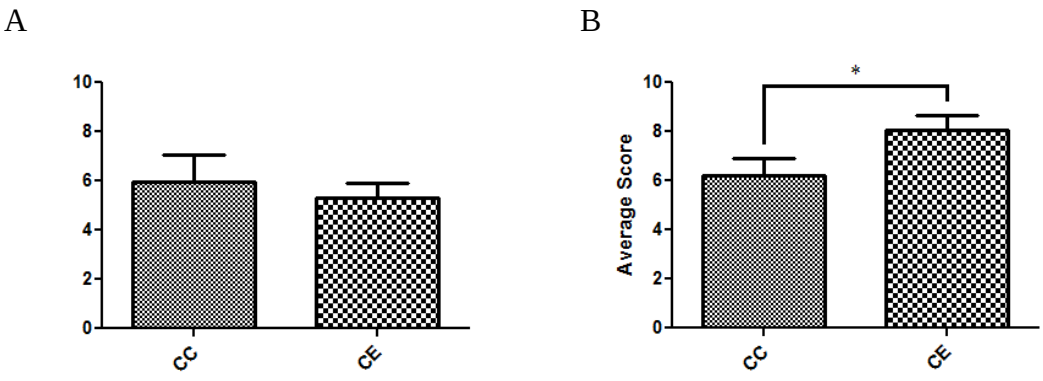

Figure 10: Number of closed arm entries in the elevated plus maze (EPM) for chronic control (CC) and chronic ethanol (CE) groups. (A) Adult CC and CE animals did not differ in the number of closed arm entries. (B) Aged CE animals made significantly more closed arm entries than CC. * $p < 0.05$, error bars denote SEM.

Open Field

Adult: Distance traveled in meters in the open field was similar between adult rats treated with chronic ethanol and those treated with chronic control diet, $t(30) = 0.79$, $p > 0.05$ (Figure 11a). Similarly, chronic drug did not influence the percent of time in thigmotaxis, $t(30) = 0.46$, $p > 0.05$ (Figure 12a), or the number of rears, $t(14) = 0.34$, $p > 0.05$ (data not shown).

Aged: Animals treated with chronic ethanol exhibited significantly longer path lengths than controls, $t(35) = 1.95$, $p < 0.05$ (Figure 11b). There was no significant effect of chronic drug treatment on percent of time spent in thigmotaxis, $t(35) = 0.04$, $p > 0.05$ (Figure 12b). Further, the number of rears was comparable between aged animals receiving ethanol and controls, $t(35) = 1.11$, $p > 0.05$ (data not shown).

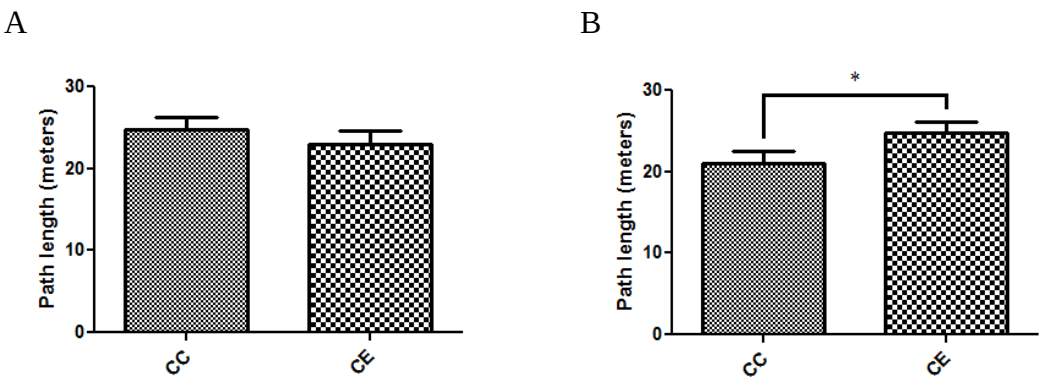

Figure 11: Path length in the open field (OF) for chronic control (CC) and chronic ethanol (CE) groups. (A) Adult CC and CE animals did not differ in distance travelled. (B) Aged CE animals had significantly longer path lengths than CC animals. * $p < 0.05$, error bars denote SEM.

A B

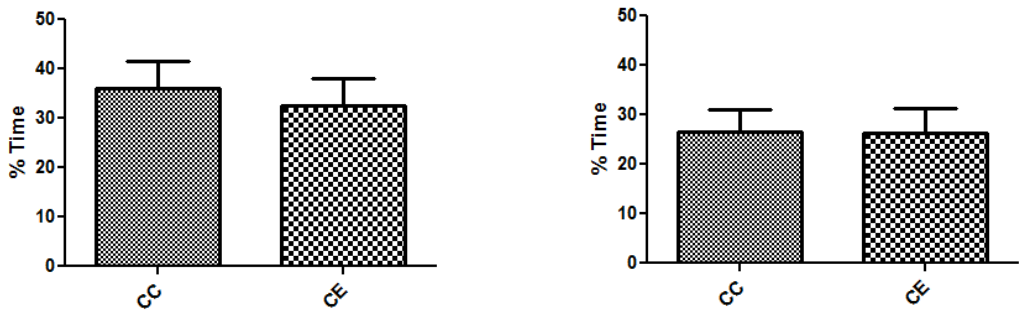

Figure12: Percent time spent in thigmotaxis in the open field (OF) for chronic control (CC) and chronic ethanol (CE) groups. (A) Adult CC and CE animals did not differ in thigmotaxis. (B) Aged CC and CE animals did not differ in thigmotaxis. Error bars denote SEM.

Morris Water Maze Training

Adult: Animals learned the location of the hidden platform as evidenced by significant main effects of day for latency, $F(5, 150) = 40.61$, $p < 0.001$, and path length, $F(5, 150) = 30.54$, $p < 0.001$. Additionally, adult animals treated with chronic ethanol had increased latency, $F(1, 150) = 5.84$, $p < 0.05$ (Figure 13a), and path length, $F(1, 150) = 5.07$, $p < 0.05$ (Figure 14a), compared to controls during training. A significant interaction between day and chronic drug was found for swim speed, $F(5, 150) = 4.54$, $p < 0.001$, and Bonferroni post-tests indicated that adults treated with chronic ethanol had slower swim speed than controls specifically during day three of training (Figure 15a). Finally, the percentage of time spent in thigmotaxis decreased across time $F(5, 150) = 75.18$, $p < 0.001$, but did not differ by chronic drug treatment, $F(1, 150) = 2.47$, $p > 0.05$, (data not shown).

Aged: A significant interaction between day and chronic drug was found for aged rats in latency, $F(5, 170) = 3.05$ $p < 0.05$. However, Bonferroni post-tests failed to identify any comparisons that were significant at the $p < 0.05$ level. Aged animals learned

the location of the platform as revealed by decreases in path length across training days, $F(5, 170) = 19.96$, $p < 0.001$. Chronic drug treatment did not influence learning as assessed by either latency, $F(5, 170) = 0.84$, $p > 0.05$ (Figure 13b), or path length, $F(1, 170) = 0.90$, $p > 0.05$ (Figure 14b).

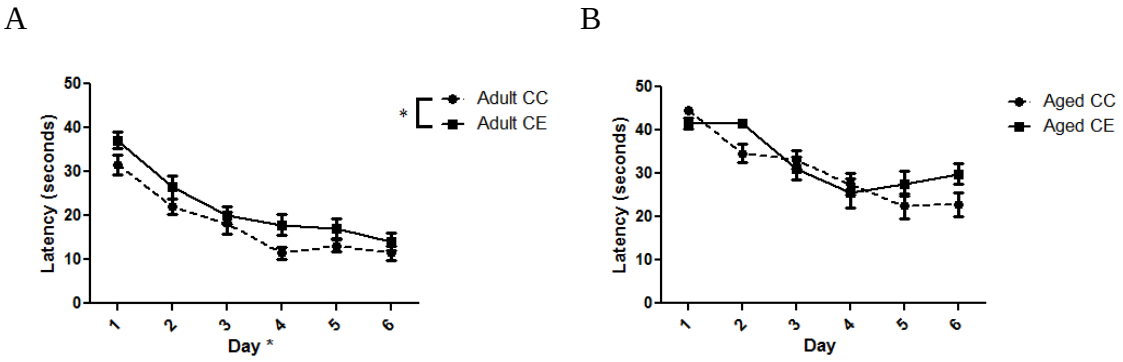

Figure 13: Latency during water maze training for chronic control (CC) and chronic ethanol (CE) groups. (A) Adult CE animals had impaired latency to the platform compared to controls. (B) A significant interaction between day and chronic drug treatment was found for aged animals. * $p < 0.001$, error bars denote SEM.

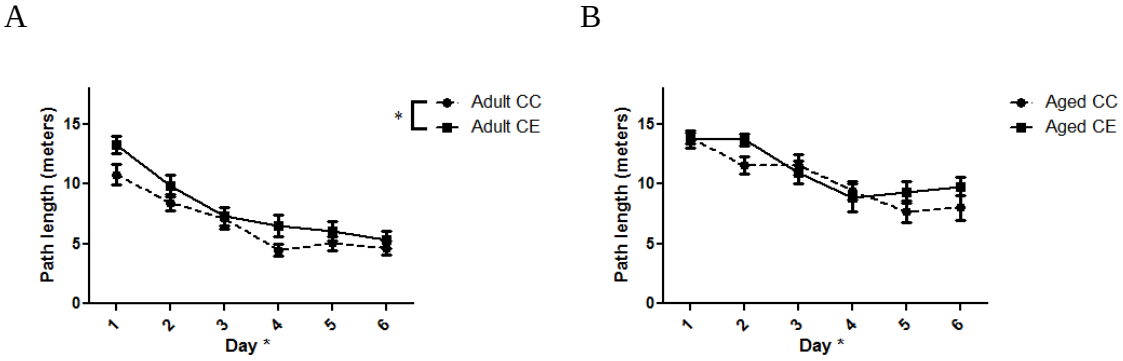

Figure 14: Path length during water maze training for chronic control (CC) and chronic ethanol (CE) groups. (A) Adult CE animals had increased path lengths to the platform compared to controls. (B) Aged CC and CE animals did not differ in path lengths. * $p < 0.05$, error bars denote SEM.

In aged animals, swim speed decreased across time, $F(5, 170) = 3.99$, $p < 0.01$, but did not differ by chronic drug treatment, $F(1, 170) = 0.02$, $p > 0.01$ (Figure 15b). Similarly, time spent in thigmotaxis was comparable between aged animals treated with chronic ethanol and controls, $F(1, 170) = 2.55$, $p > 0.05$ (data not shown).

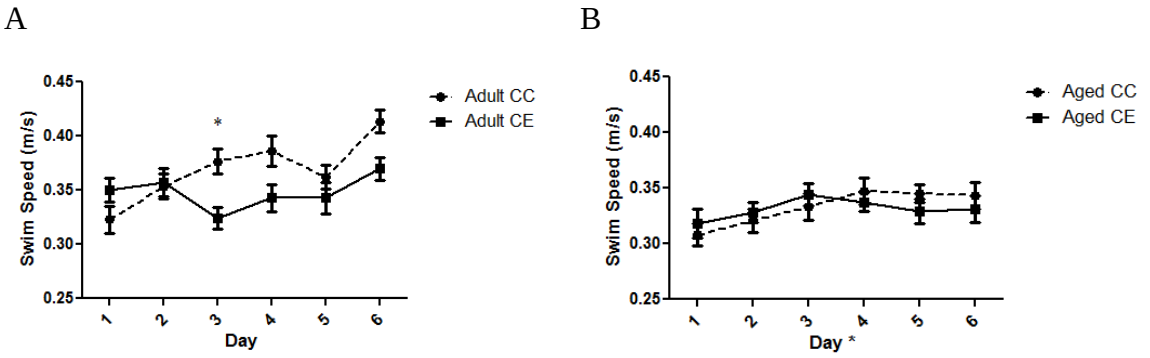

Figure 15: Swim speed during water maze training for chronic control (CC) and chronic ethanol (CE) groups. (A) Adult CC animals had increased swim speed compared to CE rats during day three of training. (B) Aged CC and CE animals did not differ in swim speed.* $p < 0.05$, error bars denote SEM.

Ethanol Test

Aerial Righting Reflex (ARR)

Adult: Animals treated with acute ethanol required significantly higher heights for successful righting compared to saline controls, $F(1,28) = 33.72$, $p < 0.001$ (Figure 16a). However, no significant main effects of chronic drug treatment were found, $F(1,28) = 0.50$, $p > 0.05$.

Aged: Similarly, a significant main effect of acute drug indicated that aged animals receiving acute ethanol had impaired righting performance compared to controls, $F(1,31) = 443.40$, $p < 0.001$ (Figure 16b). Chronic drug treatment did not influence successful righting in aged animals, $F(1,31) = 00.00$, $p > 0.05$.

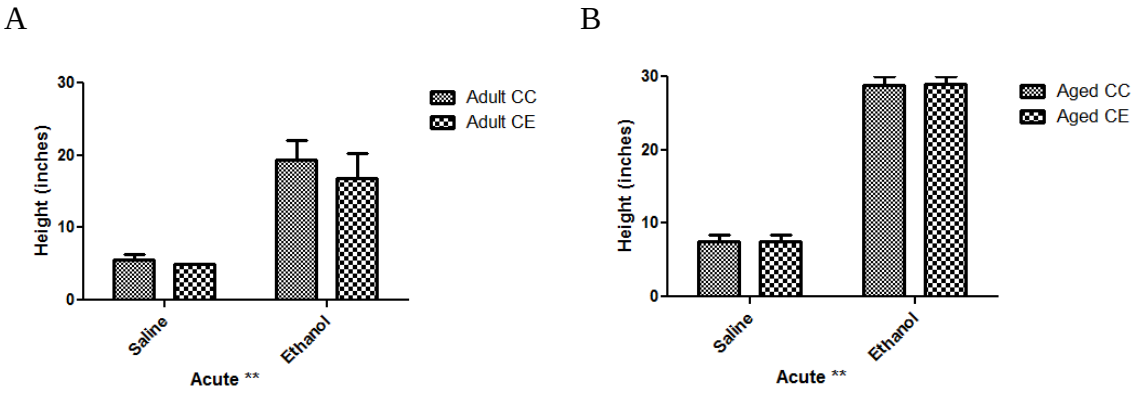

Figure 16: Height required for successful righting for chronic control (CC) and chronic ethanol (CE) groups. (A) Adult animals treated with acute ethanol required a higher height for successful righting. (B) Adult animals treated with acute ethanol required a higher height for successful righting. ** $p < 0.001$, error bars denote SEM.

Water Maze Testing

Adult: Acute ethanol impaired spatial memory as evidenced by significant main effects as measured by latency, $F(1, 28) = 27.38$, $p < 0.001$ (Figure 17a), and path length, $F(1, 28) = 18.92$, $p < 0.001$ (Figure 18a). No significant main effects of chronic drug treatment were found for either latency, $F(1, 28) = 0.04$, $p > 0.05$, or path length, $F(1, 28) = 0.63$, $p > 0.05$. A significant interaction between acute and chronic drug was found for swim speed, $F(1, 28) = 5.92$, $p < 0.05$, and Bonferroni post-tests indicated that, among adults treated with chronic control diet, animals receiving acute ethanol swam slower than those that received saline (Figure 19a). Similarly, adults treated with acute ethanol exhibited greater thigmotaxis during testing than did saline controls, $F(1, 28) = 9.16$, $p < 0.01$ (data not shown).

Aged: Animals treated with acute ethanol had higher latency to the platform than saline controls, $F(1, 31) = 6.28$, $p < 0.05$ (Figure 17b). The main effect of path length was non-significant, $F(1, 231) = 0.82$, $p > 0.05$ (Figure 18b), as were all effects of chronic drug treatment. As expected, aged animals treated with acute saline swam significantly

faster than those treated with acute ethanol, $F(1, 31) = 58.75$, $p < 0.001$ (Figure 19b). Similarly, a significant main effect of acute drug was found for thigmotaxis indicating that acute ethanol increased the percent of time spent in the outer edge of the maze, $F(1, 31) = 11.03$, $p < 0.01$ (data not shown).

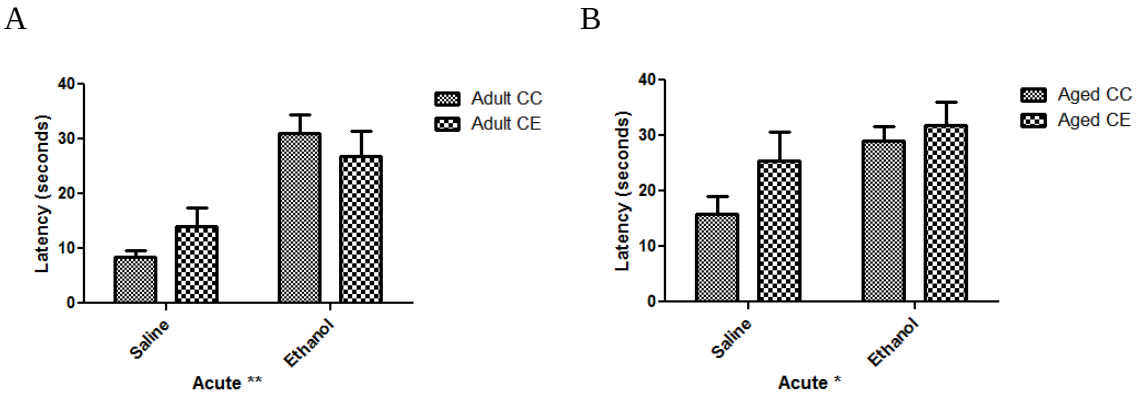

Figure 17: Latency during water maze testing for chronic control (CC) and chronic ethanol (CE) groups. (A) Adult animals treated with acute ethanol had higher latency than animals receiving acute saline. (B) Aged animals treated with acute ethanol had higher latency than animals receiving acute saline. * $p < 0.05$, ** $p < 0.001$, error bars denote SEM.

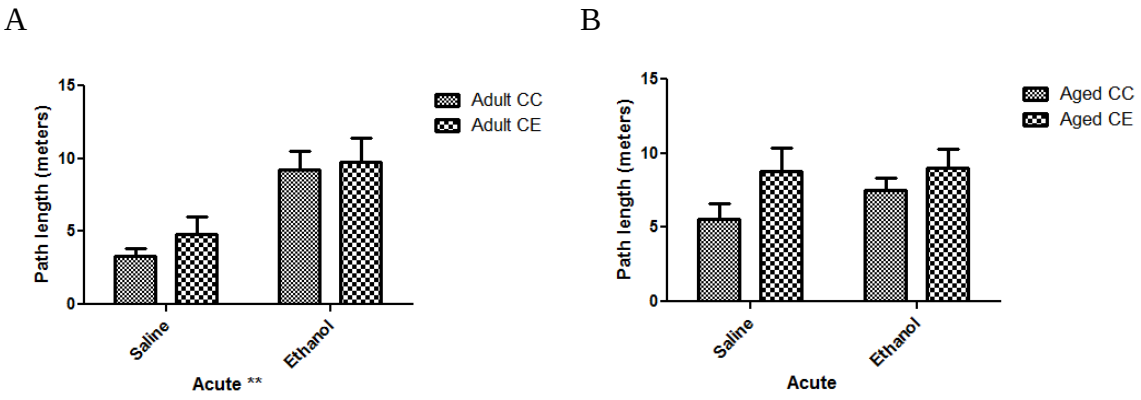

Figure 18: Path length during water maze testing for chronic control (CC) and chronic ethanol (CE) groups. (A) Adult animals treated with acute ethanol had longer path lengths than animals receiving acute saline. (B) Acute drug did not influence path lengths in aged animals. ** $p < 0.001$, error bars denote SEM.

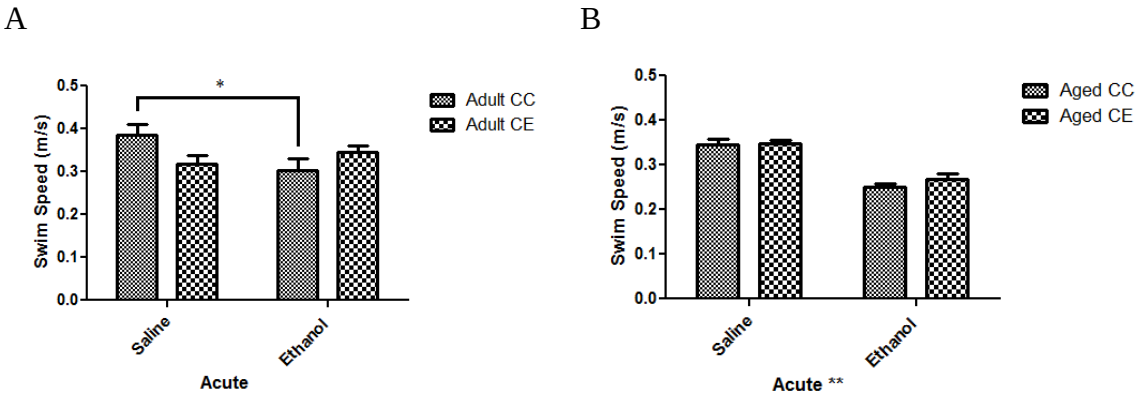

Figure 19: Swim speed during water maze testing for chronic control (CC) and chronic ethanol (CE) groups. (A) Adult CC animals treated with acute ethanol had slower swim speeds than animals receiving acute saline. (B) Aged animals treated with acute ethanol had decreased swim speed compared to saline controls. * $p < 0.05$, ** $p < 0.001$, error bars denote SEM.

BECs

Both adult and aged animals showed significant main effects of chronic drug treatment on blood ethanol concentration levels following 1.5 g/kg acute ethanol. Adults receiving chronic ethanol exhibited metabolic tolerance as evidenced by significantly lower BECs following acute ethanol administration than chronic controls, $t(1, 14) = 1.91$, $p < 0.05$ (Figure 20a). Similarly, aged chronic-ethanol exposed animals had lower BECs in response to acute ethanol than aged chronic controls, $t(1, 17) = 4.75$, $p < 0.001$ (Figure 20b).

MWM Reversal Paradigm

Adult: Animals learned the new location of the platform as evaluated by latency, $F(2, 60) = 6.65$, $p < 0.01$, and path length, $F(2, 60) = 5.95$, $p < 0.01$. Furthermore, significant main effects of chronic drug for both latency, $F(1, 60) = 6.91$, $p < 0.05$ (Figure 21a), and path length, $F(1, 60) = 4.25$, $p < 0.05$ (Figure 22a), revealed that chronic ethanol consumption impaired spatial learning in the cognitively-challenging reversal

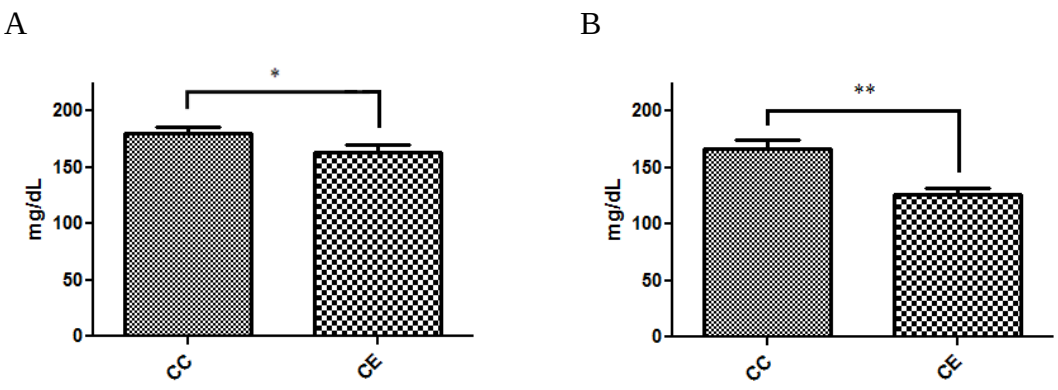

Figure 20: Blood ethanol concentration (BEC) levels following ethanol testing for chronic control (CC) and chronic ethanol (CE) groups. (A) Adult CE animals had significantly lower BECs than CC animals. (B) Aged CE animals had significantly lower BECs than CC animals. * $p < 0.05$, ** $p < 0.001$, error bars denote SEM.

paradigm. Similarly, chronic ethanol-treated animals spent a greater percentage of time in thigmotaxis, $F(1, 60) = 14.31$, $p < 0.001$ (data not shown). There was no significant difference in swim speed between animals treated with chronic ethanol or control diet, $F(1, 60) = 2.57$, $p > 0.05$ (data not shown).

Aged: Performance of aged animals did not differ across time as indicated by non-significant main effects of training day for latency, $F(2, 64) = 2.32$, $p > 0.05$, and path length, $F(1, 64) = 1.81$, $p < 0.05$. However, animals treated with chronic ethanol diet did show impaired spatial learning as assessed by both latency, $F(1, 64) = 6.85$, $p < 0.05$ (Figure 21b), and path length, $F(1, 64) = 7.35$, $p < 0.05$ (Figure 22b). Additionally, aged rats treated with ethanol diet had increased thigmotaxis during learning compared to controls, $F(1, 64) = 6.90$, $p < 0.05$ (data not shown). Chronic drug treatment did not influence swim speed during the reversal paradigm, $F(1, 64) = 0.09$, $p > 0.05$ (data not shown).

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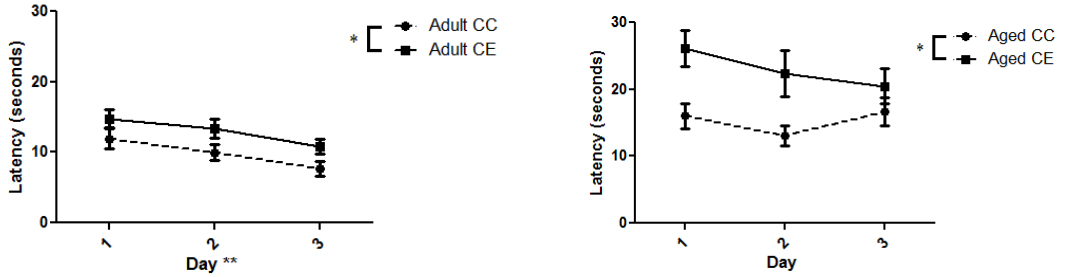

Figure 21: Latency in the water maze reversal paradigm for chronic control (CC) and chronic ethanol (CE) groups. (A) Adult CE animals had increased latency to the platform compared to controls. (B) Aged CE had increased latency to the platform compared to controls. * $p < 0.05$, ** $p < 0.01$, error bars denote SEM.

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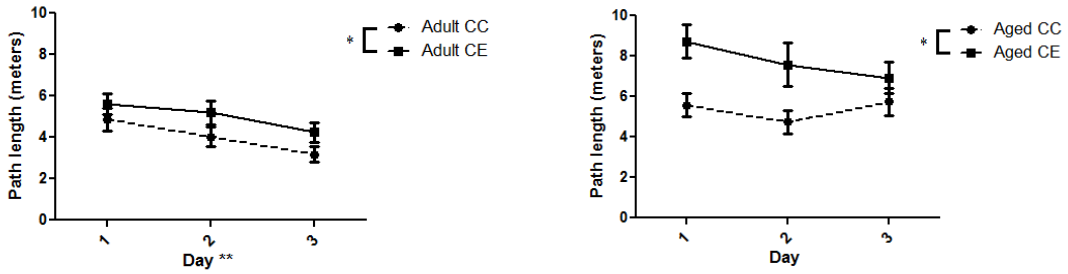

Figure 22: Path length in the water maze reversal paradigm for chronic control (CC) and chronic ethanol (CE) groups. (A) Adult CE animals had significantly longer path lengths compared to controls. (B) Aged CE animals had significantly longer path lengths compared to controls. * $p < 0.05$, ** $p < 0.01$, error bars denote SEM.

Allopregnanolone Test

Adult: Compared to the previous day of training, allopregnanolone impaired performance of adult animals in the water maze as measured by latency to the platform, $F(1, 28) = 10.80$, $p < 0.01$ (Figure 23a). There was a non-significant trend for path length to increase under allopregnanolone, $F(1, 28) = 3.72$, $p = 0.06$ (data not shown). Performance was not mediated by chronic drug diet or the recent acute drug challenge for either latency or path length. Looking specifically at spatial memory 20 minutes

following administration of allopregnanolone, main effects for chronic drug, $F(1, 28) = 0.11$, $p > 0.05$, and recent acute drug, $F(1, 28) = 3.35$, $p > 0.05$, were non-significant for latency (Figure 24a). Path length measures were similarly non-significant for chronic drug, $F(1, 28) = 0.02$, $p > 0.05$, and recent acute drug, $F(1, 28) = 2.66$, $p > 0.05$ (data not shown). However, adults treated with acute ethanol during testing had increased thigmotaxis under allopregnanolone compared to those that received saline, $F(1, 28) = 12.03$, $p < 0.01$ (Figure 25a).

Aged: Animals did not show impaired spatial memory under allopregnanolone as evidenced by non-significant effects for latency, $F(1, 30) = 1.02$, $p > 0.05$ (Figure 23b), and path length, $F(1, 30) = 0.03$, $p > 0.05$. However, when data from the allopregnanolone test day was analyzed, a significant main effect of prior acute drug was found for latency, indicating that animals receiving acute ethanol during the ethanol challenge had greater spatial memory impairments under allopregnanolone than saline controls, $F(1, 30) = 4.64$, $p < 0.05$ (Figure 24b).

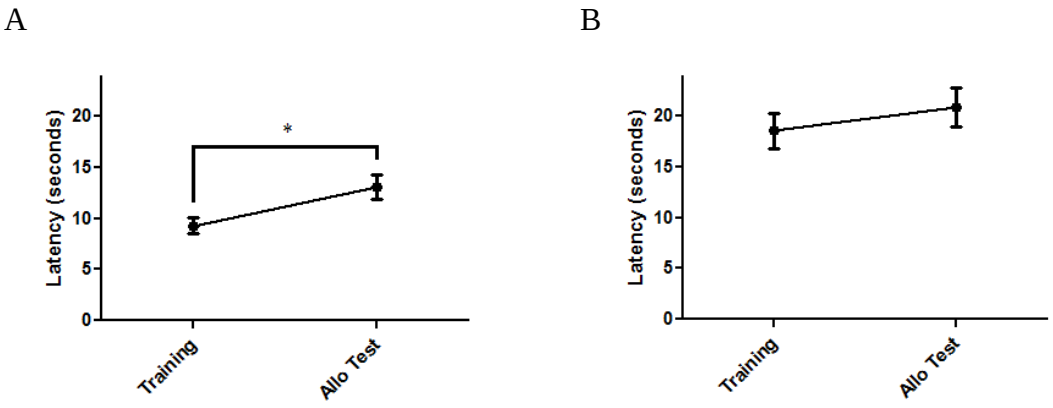

Figure 23: Latency in the water maze during the final day of training compared to the allopregnanolone test. (A) Adult animals had increased latency to the platform when challenged with allopregnanolone. (B) Aged animals showed no difference in latency to the platform when treated with allopregnanolone. * $p < 0.01$, error bars denote SEM.

Neither chronic drug nor acute drug mediated allopregnanolone-induced performance as assessed by path length (data not shown), or thigmotaxis (Figure 25b).

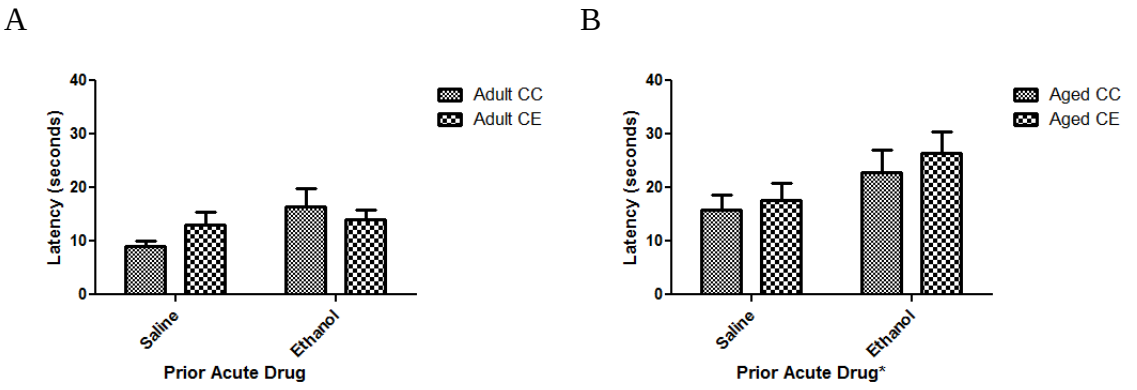

Figure 24: Latency in the water maze during allopregnanolone testing for chronic control (CC) and chronic ethanol (CE) groups. (A) Neither chronic drug treatment nor the recent drug challenge influenced latency to the platform for adults. (B) Aged animals receiving acute ethanol during ethanol testing had increased latency to the platform compared to those receiving acute saline. * $p < 0.05$, error bars denote SEM.

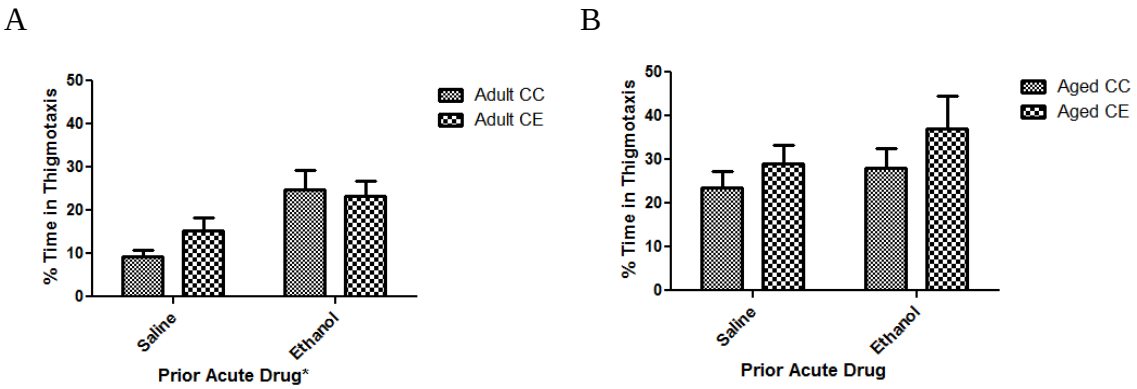

Figure 25: Thigmotaxis in the water maze during allopregnanolone testing for chronic control (CC) and chronic ethanol (CE) groups. (A) Adult animals receiving acute ethanol during ethanol testing had increased thigmotaxis compared to those receiving acute saline. (B) Neither chronic drug treatment nor the recent drug challenge influenced thigmotaxis for aged animals. (B) * $p < 0.01$, error bars denote SEM.

CHAPTER FOUR

Discussion

The results from the present study add to the growing body of evidence examining the effects of chronic ethanol consumption and withdrawal in aged rats. Findings indicate that the aged are uniquely sensitive to ethanol withdrawal-induced anxiety, and that both adult and aged rats show cognitive deficits following repeated periods of ethanol intoxication and withdrawal. Although aged animals consumed more ethanol diet than adults, they consumed less g/kg ethanol relative to their large body size. As such, adult animals had significantly higher BECs and experienced more severe ethanol withdrawal symptoms on a standardized rating scale than aged rats. Nonetheless, aged animals only were sensitive to anxiety-like behavior and hyperactivity in both the elevated plus maze and open field during withdrawal. In accordance with previous studies, we have provided further evidence that chronic ethanol consumption impairs spatial memory in the water maze in both adult and older age groups. Finally, we investigated the effects of an acute dose of allopregnanolone on spatial memory in adult and aged rats. Performance of adult animals, but not aged, was adversely affected by allopregnanolone in the water maze. Additionally, an acute ethanol challenge exacerbated allopregnanolone-induced cognitive deficits in both age groups.

Liquid ethanol has reliably been used to initiate ethanol dependence and associated physical withdrawal symptoms in adult rodents (Devaud et al., 2002; Erden et al., 1999; File et al., 1993; Gatch, Wallis, & Lal, 1999; Lal et al., 1991; Moy et al., 1997; Wood et al., 1982). However, to the best of our knowledge, this is the first study to use

liquid ethanol diet to investigate ethanol withdrawal specifically in aged rats. However, Wood et al. (1982) used 14 days of five percent liquid ethanol diet to successfully examine ethanol withdrawal syndrome in male C57BL/6NNIA mice aged three, 14, and 25 months. Although the older mice consumed less ethanol per body weight than three-month-old animals, all mice consumed at least 14 g/kg ethanol by the end of treatment. Importantly, older mice were found to be significantly more intoxicated on a standardized scale and experienced greater withdrawal- induced body tremors than did the younger group (Wood et al., 1982). Unfortunately, aged rats also consumed less ethanol by weight than did young adults in the present study. Whereas the weight difference between age groups of mice was less than eight grams, approximately, in Wood et al. (1982), aged rats weighed over 170 grams more than adults in the present study. Consequently, adult rats consumed approximately 9.7 g/kg ethanol while aged animals consumed only 7.2 g/kg ethanol. Thus, the large weight difference between age groups likely contributed to decreased ethanol consumption and diminished scores on the standardized withdrawal scale in aged rats. Given that liquid ethanol diet is the most commonly used procedure to induce dependence in rats (Kliethermes, 2005), these findings have striking implications for future investigations of ethanol dependence and withdrawal in older rodents. Perhaps alternate ethanol administration methods such as ethanol vapor chambers, i.p. injection, or intragastric gavage would produce more similar ethanol consumption levels between adult and aged rats and allow for a better comparison of ethanol withdrawal syndrome.

Limited research with human alcoholics suggests that the elderly may experience more severe alcohol withdrawal symptoms than younger drinkers. Liskow et al. (1989) reported that older drinkers (ages 58 to 77) had greater withdrawal symptom ratings and

required higher doses of medication during treatment than younger individuals (ages 21 to 33). Similarly, Brower et al. (1994) likewise found that elderly drinkers showed greater withdrawal symptoms that persisted for a longer duration compared to younger patients. As expected, older adults had a longer history of problem drinking than younger adults, but there was no difference in the number of drinks consumed per day, number of drinking days in the previous month, or the number of prior hospitalizations (Brower et al., 1994). Elderly patients may also be more likely to experience severe symptoms including cognitive impairment, falls, and delirium during withdrawal (Kraemer et al., 1997). However, a contrasting study reported that the severity of ethanol withdrawal scores, as well as the amount and duration of medication administered during treatment, did not differ by age (Willinger et al., 2002). Thus, inconsistent reports from both human and rodent investigations highlight the need for further examination of ethanol withdrawal in the aging population.

The present study utilized a four-item rating scale that has previously been used to assess ethanol withdrawal in adult rats (Clemmesen & Hemmingsen, 1984; Hemmingsen et al., 1984; Riihioja et al., 1999). Although young adults achieved similar withdrawal scores as observed in previous research, scores for aged rats were considerably lower. The most obvious explanation for decreased withdrawal ratings in aged rats is the difference in ethanol consumed per body weight throughout the liquid ethanol diet treatment in adult versus aged rats. The ethanol consumed by adult rats was in agreement with previous values that have been shown to initiate dependence using liquid diet (Devaud et al., 1995b; Morrow et al., 1992), but aged animals consumed considerably less ethanol during treatment. Perhaps the quantity of daily ethanol consumed by the aged

rats was insufficient to produce marked withdrawal symptoms as assessed by the four-item rating scale. Alternately, the utilized rating system may be more appropriate for use with adult rodents than aged. The original withdrawal measure proposed by Hemmingsen, Clemmensen, and Barry (1984) included eight categories, but subsequent item analysis revealed that four items were most valid in identifying severity of withdrawal (rigidity, hypoactivity, intentional tremor, and irritability). Notably, the validity of this measure was tested in young adult male Wistar rats 220 grams at arrival. Aged animals may exhibit unique characteristics of ethanol withdrawal that are better represented by separate and different measures than are used with younger adult animals. Indeed, older human alcoholics present with different symptoms of ethanol withdrawal than younger adults (Dar, 2006), and current diagnostic measures commonly underrepresent alcohol use problems in the elderly (Adams, Barry, & Fleming, 1996; Graham, 1986; Patterson & Jeste, 1999). Despite the absence of robust withdrawal signs in aged rats using the four-item scale, the amount of ethanol consumed appears to be sufficient to initiate withdrawal syndrome as aged rats exhibited anxiety-like behavior and hyperactivity during ethanol withdrawal.

Aged rats appear to be particularly vulnerable to anxiety-like behavior during withdrawal. As aged animals consumed less ethanol during chronic treatment than adults, withdrawal-induced anxiety may be more pronounced or manifest with lower amounts of ethanol consumption in the aged. Previous work demonstrated that older six-and-a-half month rats were more sensitive to withdrawal-induced hypoactivity in the open field compared to younger three month animals (Maier & Pohorecky, 1989). However, to the best of our knowledge, the current project is the first to assess withdrawal-induced

anxiety specifically in aging rats. The role of anxiety in alcohol dependence and abuse has been a well-studied area of interest among clinicians and researchers. Preclinical investigations report that rats with high baseline anxiety levels show greater ethanol preference and voluntary intake than less anxious animals (Spanagel et al., 1995). Similarly, trait anxiety has been shown to be a significant predictor of relapse drinking in detoxified male and female humans patients (Willinger et al., 2002). Importantly, anxiety disorders and alcohol use disorders show extreme comorbidity (Grant et al., 2004); individuals with any diagnosed anxiety disorder reportedly have a 50 percent increased risk of also being diagnosed with an alcohol disorder (Kushner, Abrams, & Borchardt, 2000). Furthermore, alcohol-dependent patients with co-existing anxiety disorders have more severe symptoms of alcohol withdrawal (Johnston et al., 1991) and higher rates of relapse compared to human drinkers without comorbid disorders (Driessen et al., 2001). Although the relationship between anxiety and AUDs is not clearly defined, several theories have been posited. It has been hypothesized that anxiety disorders may promote pathological alcohol use by alleviating anxiety, that chronic alcohol consumption and withdrawal may promote anxiety, or that a third factor contributes to both anxiety and alcohol disorders (Kushner et al., 2000; Smith & Randall, 2012). Expectedly, withdrawal-induced anxiety has been identified as a significant motivational factor that may lead to relapse in humans (for review, see Becker, 2014). Given the susceptibility aged rats show to anxiety-like behavior during ethanol withdrawal, treatment options targeting anxiety during the withdrawal syndrome may prove especially efficacious for elderly drinkers.

Several mechanisms have been implicated in the anxiogenic symptoms associated with ethanol withdrawal. Corticotropin-releasing factor (CRF) appears to play an

important role in dependence and withdrawal associated with many drugs of abuse, including alcohol (Logrip, Koob, & Zorrilla, 2011). Acute alcohol activates the HPA axis and stimulates CRF production from the hypothalamus (Rivier, Bruhn, & Vale, 1984) and ethanol preference and exposure has been shown to influence brain CRF levels. For instance, CRF concentrations are decreased in alcohol-preferring rats in the hypothalamus, amygdala, prefrontal and cingulate cortices compared with non-preferring rats (Ehlers et al., 1992). Additionally, rats exposed to seven days of ethanol vapor also show decreased hypothalamic CRF levels, suggesting that chronic ethanol results in greater CRF release from the median eminence (Rivier et al., 1984). Given the well-known role of CRF in behavioral and physiological responses to stress, CRF has been implicated in the anxiogenic effects associated with ethanol withdrawal (Menzaghi et al., 1994). Accordingly, intracerebroventricular (ICV) administration of a CRF antagonist attenuates anxiety-like behavior during ethanol withdrawal as evaluated by open arm time and entries in the elevated plus maze in ethanol-dependent rats (Baldwin et al., 1991).

In particular, the amygdala and related structures have been identified as an important area of interest underlying CRF neurotransmission and withdrawal-induced anxiogenesis (Menzaghi et al., 1994). Rats made dependent on liquid ethanol diet show reduced CRF-like immunoreactivity in the amygdala and hippocampus during day one of ethanol withdrawal, and CRF levels become elevated six weeks post-withdrawal specifically in the amygdala (Zorrilla, Valdez, & Weiss, 2001). Similarly, extracellular CRF levels are also increased in the amygdala *in vivo* at the peak of ethanol withdrawal (Pich et al., 1995). Furthermore, withdrawal-induced extracellular CRF levels are

increased in the bed nucleus of the stria terminalis (BNST), an area which receives CRF-containing projections from the central amygdala, and these elevations are reversed following access to liquid ethanol diet (Olive et al., 2002). Importantly, microinjection of a CRF antagonist directly into the central nucleus of the amygdala reverses anxiogenic-like behavior in the elevated plus maze in rats undergoing ethanol withdrawal (Rassnick et al., 1993). The CRF system also appears to play a role in the motivational effects of ethanol dependence and withdrawal. ICV administration of D-Phe-CRF, a CRF antagonist, reverses withdrawal-induced increases in operant ethanol self-administration (Valdez et al., 2002). Likewise, bilateral infusion of the CRF receptor antagonist into the central nucleus of the amygdala also decreases voluntary limited-access consumption of ethanol solution in mice following intermittent ethanol vapor exposure (Finn et al., 2007). Given age-related changes in the HPA axis functioning, the elderly may be particularly susceptible to ethanol-induced disturbances in the stress response.

Research with rodents has demonstrated that aging is associated with altered HPA axis function which can lead to impaired stress response and exacerbate the effects of chronic ethanol and aging (Spencer & Hutchison, 1999). Compared to younger rats, aged animals show similar stress response magnitudes and corticosterone secretion during chronic stress. However, aged rats exhibit increased basal corticosterone levels and show a slower return to baseline following stress, suggesting that age may result in the impaired ability to recovery and adapt to stressful events (Sapolsky, Krey, & McEwen, 1983). Additionally, repeated ethanol exposure typically results in habituation to ethanol-induced corticosterone secretion, but evidence indicates that aged animals show an impaired ability to develop tolerance to ethanol-induced corticosterone levels compared

younger adults (Spencer & McEwen, 1997). Aged rats also show impaired ACTH suppression following a dexamethasone challenge compared to younger animals, suggesting age-related impairments in the negative feedback loop (Hatzinger et al., 1996). Thus, the HPA axis appears to become highly dysregulated with aging. As CRF plays a major function in regulation of the HPA axis (Smith & Vale, 2006), age-related differences in CRF expression may underlie altered responses to alcohol-induced acute and chronic stress. Aging alone is characterized by a decrease in CRF content and mRNA levels in the amygdala, BNST, and paraventricular hypothalamus (Cizza et al., 1994; Kasckow et al., 1999). Further, aged rats show elevated CRF peptide levels in the paraventricular nucleus following a chronic intermittent stress paradigm compared to younger animals (Herman et al., 2001). Importantly, aged rats also show decreased quantities of CRF-binding protein in the basolateral nucleus and decreased CRF-like immunoreactivity in the central nucleus of the amygdala compared to four-month old rats. These age-related changes in the amygdala CRF system likely contribute to impaired stress response, and possibly cognitive decline, in aging (Xiao et al., 2006). Future research should investigate CRF in the amygdala following chronic ethanol exposure as a potential mechanism underlying age-related vulnerability to withdrawal-induced anxiety.

Results from the present study provide further evidence demonstrating that chronic ethanol and withdrawal impair spatial learning in rodents. Investigations have shown that chronic ethanol or withdrawal impairs cognitive functioning using a variety of tasks (Arendt et al., 1989; Baydas et al., 2005; Franke et al., 1997; Lukoyanov et al., 1999; Santucci et al., 2004; Walker & Hunter, 1978), while other investigations have seen no significant cognitive deficits following chronic ethanol treatment (Blokland et al.,

1993; Boulouard et al., 2002; Maier & Pohorecky, 1987). Young adult animals treated with chronic ethanol exhibited increased latency and path length to the water maze platform during the initial six days of water maze acquisition compared to controls. However, chronic ethanol did not significantly impair initial water maze learning in aged animals. We speculate that the decreased learning rate observed in older animals may have created a floor effect such that chronic ethanol was not able to result in pronounced impairments compared to older controls. Importantly, both ethanol-dependent adult and aged animals showed learning deficits during the cognitively challenging reversal paradigm. Increased thigmotaxis in the maze may reflect an inefficient search strategy during swimming (Chin et al., 2011) which may partially explain spatial impairments observed in ethanol-treated animals during the reversal paradigm. The present findings agree with a previous study similarly reporting deficits during reversal learning in ethanol-exposed animals and suggest that chronic ethanol may impair behavioral flexibility (Santín et al., 2000). Future work should utilize more complex tasks to fully detect cognitive deficits in rodents treated with chronic ethanol.

Although adult and aged animals treated with chronic ethanol showed metabolic tolerance in response to an acute ethanol challenge, no evidence of cognitive tolerance was observed in the water maze. Previous work has shown that chronic intermittent ethanol produces tolerance to ethanol's spatial-impairing effects (Boulouard et al., 2002; Silvers et al., 2006), but functional tolerance is transient. Silvers et al. (2006) administered 5.0 g/kg ethanol or saline to adolescent rats every other day between PD 30 to PD 48 as animals were trained in the water maze on non-injection days. Groups were counterbalanced to receive 2.0 g/kg ethanol or saline on either PD 50 or PD 62 thirty

minutes prior to spatial memory testing in the water maze. CIE exposure during adolescence attenuated ethanol-induced cognitive impairments in the maze at PD 50, but not PD 62. Thus, only twelve days into ethanol withdrawal, spatial performance was similar between animals that received chronic ethanol and those that received chronic saline, indicating that tolerance was quickly reversed. Tolerance to ethanol-induced spatial deficits has been attributed to differences in allopregnanolone levels following chronic ethanol exposure (Morrow et al., 2001). Animals treated with CIE in Silvers et al. (2006) showed reduced ethanol-induced allopregnanolone levels in the hippocampus at PD 50 compared to those receiving chronic saline. Therefore, tolerance to allopregnanolone may contribute to reduced ethanol-induced cognitive deficits following chronic ethanol treatment. However, Silvers et al. (2006) reported that CIE exposure led to increases in allopregnanolone levels in adolescents following acute ethanol administration at PD 62, possibly indicative of hyperactivity to a future stress response (Silvers et al., 2006). Similar to the current investigation, pervious work has likewise observed dissociation between functional tolerance and metabolic tolerance. Van Skike et al. (2012) treated adolescent rats with four days of 16 hours ethanol vapor following water maze training and found that chronic ethanol-treated animals exhibited reduced BEC levels in response to acute ethanol, but showed similar spatial memory impairments during water maze testing, compared to control animals. The authors similarly speculate that metabolic tolerance, but the lack of overt cognitive tolerance, may be explained by the memory-impairing effects of ethanol-induced hippocampal allopregnanolone (Van Skike et al., 2012). However, it is critical to emphasize that investigation of metabolic tolerance, cognitive tolerance, and ethanol-induced allopregnanolone levels following

chronic ethanol treatment has only been observed in adolescent and young adult rats. Future work should explore the associations between ethanol tolerance and allopregnanolone in older adult and aged rodents to reveal potential age-related mechanisms contributing to differences in ethanol-induced cognitive deficits.

In contrast to our initial hypothesis, chronic ethanol did not result in tolerance or pronounced deficits in motor performance as measured by aerial righting reflex. Perhaps tasks that assess fine motor coordination, such as the accelerating rotarod, would be a better indicator of motor deficits caused by chronic drinking in rodents. Servais et al. (2005) treated mice with ethanol solution as the sole source of fluids for three months and observed that 18 percent ethanol resulted in impairments in accelerating rotarod and runway performance in a naïve task. Impairments in motor coordination were attributed to deficits in cerebellar output as ethanol-treated animals also exhibited significant decreases in Purkinje cell firing, the sole output of the cerebellar cortex (Servais et al., 2005). In contrast, rats given access to 20 percent ethanol for thirty minutes a day for 18-months failed to exhibit any motor impairments on the balance beam task (Baird et al., 1998). The dose and duration of ethanol exposure, as well as differences in task and species, likely contribute to inconsistent reports of ethanol-induced motor deficits in rodents.

In accordance with previous research, allopregnanolone impairs spatial memory in the water maze in young adult rats (Chin et al., 2011; Matthews et al., 2002). However, contrary to our hypotheses, aged rats showed only a non-significant trend towards impairments in spatial memory when administered allopregnanolone as compared to the previous day of training. Interestingly, no effect of chronic drug treatment was found for

performance following acute allopregnanolone administration in either adult or aged animals. Allopregnanolone has been implicated in the formation of tolerance to ethanol-induced spatial memory impairments (Morrow et al., 2001; Silvers et al., 2006). As no evidence of cognitive tolerance was observed in the water maze during ethanol testing, comparable performance between chronic ethanol and chronic control animals in response to acute allopregnanolone appears logical. Chronic ethanol-receiving groups are similarly impaired by acute ethanol in the water maze three weeks post withdrawal, and likewise show comparable impairments in the maze when challenged with acute allopregnanolone four days later. Furthermore, we identified a recent ethanol challenge as a significant contributor to allopregnanolone-induced spatial memory deficits. Aged animals treated with a previous injection of acute ethanol had increased latency to the platform when administered allopregnanolone compared to animals receiving acute saline. Furthermore, adults that received acute ethanol during testing exhibited increased thigmotaxis in the allopregnanolone test. We hypothesize that the recent ethanol challenge acted as a stressor which compounded with allopregnanolone-induced spatial impairments to result in greater cognitive deficits. Indeed, both acute stress (Barbaccia et al., 1998; Purdy et al., 1991) and ethanol (Barbaccia et al., 1999; Morrow et al., 1999; VanDoren et al., 2000) have been shown to increase plasma and cortical allopregnanolone levels. As ethanol acts as acute stress and triggers the HPA axis (Morrow et al., 2004), further investigation is needed to explain the association between recent ethanol administration and allopregnanolone's role in cognitive function.

We have thus provided evidence that aged rats are especially vulnerable to anxiety-like behavior during withdrawal from liquid ethanol diet. Furthermore, a model

of repeated ethanol intoxications and withdrawals results in cognitive learning impairments in the water maze which are evident in both adult and aged animals approximately three weeks after cessation of ethanol. The present work provides further evidence that elderly humans may be particularly sensitive to the effects of long-term alcohol consumption and withdrawal. The synergistic association between chronic ethanol consumption and anxiety highlights an important area of investigation for future work examining chronic alcoholism and withdrawal in the aged.

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