Investigating Social Interaction of CFW Male Mice during Ethanol Withdrawal

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Abstract

Ethanol dependence has been characterized in animal models by severity of withdrawal symptoms. Escalation in drinking consumption is thought to be, at least in part, a response to alleviate withdrawal symptoms, inducing long-term dependence. Handling induced convulsions (HIC) have been a reliable way to distinguish mice that are in withdrawal and to what severity, but can only be performed late into dependence following nearly 8-10 weeks of drinking with voluntary drinking models (Goldstein, 1971). The present study attempted to model another possible withdrawal behavior, anxiety, to see if this behavior would be altered during the development of dependence, and possibly predict HIC activity in future, presumably more severe, withdrawal episodes. Outbred Swiss Webster (CFW) male mice were given a social interaction test a week before ethanol access either with a male orchidectomied or female ovariectomied stimulus in a three chamber apparatus (Moy, 2004). Subjects were then placed on an intermittent, 2-bottle choice of 20% w/v ethanol solution and water, and tested for social interaction 6-8 hours into withdrawal on withdrawal days of Weeks 1, 4 and 8. HIC scores were assessed post week 8 of testing at 4 hours and 8 hours into withdrawal, and blood was collected for BEC sampling post 1 hour of ethanol access. Animals were split into statistically significant different High and Low Drinking groups. No significant difference in duration (seconds) of interaction was found for either the female or male stimulus; however, there was a significant increase in locomotor activity across all groups, Low, High and control. The High and Low Drinking groups also had significantly higher HIC scores than the control group; no difference was found in BEC levels. These results do not necessarily conclude that anxiety-like behavior is not a

component of ethanol withdrawal, but that extent of this behavior may be contingent upon a strain's inherent sociability and sensitivity to repeated behavioral testing. Additionally, the finding on locomotor activity suggests that there might be a ethanol effect due perhaps to glutamate hyperexcitability, which is characteristic of ethanol dependence and withdrawal, or a procedural effect, as control animals were also affected, due possibly to single housing stress or repeated testing. Future studies should focus on the intersection of strain type, proclivity to become ethanol dependent, and sensitivity to behavioral testing in order to better understand what behavioral measures reveal about withdrawal behaviors.

Introduction

Alcohol use disorders have been prevalent around the world, afflicting nearly 1.7% of the global population (World Health Organization, 2003). Despite present treatment options, about 25% of alcohol abusers remain dependent on, or abusers of, alcohol the following year, 27.3% are in partial remission, and 11.8% exhibit symptoms that indicate risk of relapse (Dawson, 2005). For those seeking rehabilitation, symptoms of withdrawal, such as cravings, anxiety, and dysphoria, are cause for relapse (Koob, 2001). Animal models have been useful in studying alcohol dependence, and withdrawal after long-term alcohol dependence has been primarily characterized by convulsive activity and seizures (Goldstein and Pal, 1971). However, not much is known about other withdrawal induced behavioral changes as dependence to ethanol is developed. In mice, behavioral assessments, such as the social interaction test, have been used to study nuanced changes in behavior, such anxiety-like behavior and locomotor activity (File, 1980). The purpose of this study was to explore ethanol withdrawal following development of long-term alcohol dependence in outbred mice through possible disruptions of social interaction that may be indicative of changes in anxiety-like behavior.

Alcohol Abuse in America.

Humans have long had been producing and consuming alcohol products from the first hunter-gatherer societies through the development of the first great civilizations (Cavalieri et al, 2003). More recently in the United States, the National Institute on

Alcohol Abuse and Alcoholism (NIAAA) has been examining trends in both alcohol consumption and abuse-related disorders (Williams, 1988). Since the 1950s, consumption of alcoholic beverages (spirits, wine and beer) has been steadily increasing, and in the past decade alone, prevalence of alcohol abuse has increased from 3.03% to 4.65% (Williams, 1988; Grant, 2004). About 18 million Americans have an alcohol-related disorder, and yearly, about 80,000 people die from an alcohol-related cause (Grant, 2004; Mokdad, 2004). Alcohol consumption has also had economic costs: in 2006, it was estimated that excessive alcohol consumption had a total cost of $223.5 billion (Bouchery, 2011). In addition to medical and economic costs, alcohol abuse has many social implications as well. As a legal and widely accessible drug, alcohol has been involved in several social phenomena across a variety of demographics. Alcohol use has been associated with underage use, high-risk activity, illicit drug use, sexual abuse, traffic accidents, and has high comorbidity with depression (Langbehn et al, 2006; Durbeej et al, 2014; Fergusson et al, 1995; Beata, 2014).

Treatment options for alcohol related disorders include rehabilitation programs, abstinence, mutual support groups, medications and behavioral therapy (NIAAA). However, withdrawal symptoms resulting from alcohol dependence continue to play a large role in provoking relapse of drug use, often with increased use (Hunt et al, 1971; O'Brien, 1997). Symptoms of physical dependence include cravings, loss of control, nausea, sweating and anxiety (Hasin, 2007). In severe alcohol withdrawal, these can also be accompanied by symptoms of delirium, such as disorientation, cognitive disturbances, altered circadian rhythms, sensory changes, and may be preceded by seizures (Mainerova, 2013). Further understanding of alcohol withdrawal-induced changes in

behavior is one of many compelling reasons to study the neurobiology of alcohol dependence, and may provide insight in creating more successful treatment options.

Animal Models of Alcohol Drinking.

Though epidemiological analyses can provide data about incidence and prevalence and trends in population drinking behavior, animal models of alcohol drinking allow for a more comprehensive understanding of the neurobiological mechanisms involved in abuse and withdrawal. Animal models are useful in both genetic and pharmacological manipulations, and for studying the development of chronic dependence to drugs of abuse, including alcohol.

Original models of alcohol addiction lacked translational quality. Early studies of the 1970s utilized oral forced-administration, where ethanol was incorporated into a nutritional liquid diet inducing successful chronic dependence in mice, rats, chimpanzees, and rhesus monkeys (Freund, 1969; Ogata et al, 1972; Branchey et al, 1971; Lieber & DeCarli, 1973; Pieper et al, 1972; Pieper & Skeen, 1972). Periodic nasogastric intubation was also found to produce physical dependence to alcohol, which was notably accelerated in rats (Ellis & Pick, 1969, 1970, 1971). Intragastric and intravenous infusions of particular doses have also proven sufficient to induce dependence across several species (Mello, 1976). Inhalation of ethanol vapor has been extremely successful in rapidly producing elevated blood alcohol content to 200-300 mg/dl, and when paired with drinking protocols has caused increased drinking (Rogers et al, 1979; Becker & Lopez, 2004; Griffin et al, 2009). All these models have been useful in producing physical alcohol dependence among a variety of species, and allow researchers to know

amount of alcohol intake. However, these models are examples of forced drinking protocols, which reduces translational validity; additionally, non-oral routes of administration involve surgical procedures that also limit generalizability to a human drinker.

Behavioral models of ethanol self-administration have generally produced lower alcohol intake when compared to many forced-exposure models, but are notably more translatable to human drinkers (Mello, 1976). One of the first, schedule-induced polydipsia was developed in 1961, and involved presenting rats dry food pellets on a particular schedule, producing high intake of any liquid, including alcohol (Falk, 1961). Additionally, increasing concentration of the ethanol solution to 5-6% over the course of 12 weeks was found to induce physical dependence and maintain blood alcohol levels above 100 mg/dl; however, this also requires food restriction via schedule-induced polydipsia (Falk et al, 1972).

Intravenous self-administration, though used less infrequently, has been reported to demonstrate positive reinforcement of ethanol reward, particularly with ethanol pre-exposure, higher doses and extended self-administration training (Gauvin et al, 1992; Bienkowski et al, 1995; Biala & Kotlinska, 1999; Ikegami, 2002). Sucrose fading, in which ethanol solution and saccharin are combined in order for development of ethanol preference as sucrose is progressively removed over time, was advantageous for allowing animals to voluntarily drink to dependence at ethanol concentrations of 10 and 20% (Samson, 1986; Tolliver et al, 1988; Matthews et al, 2001).

Binge-drinking specifically has been mimicked in another voluntary drinking model known as ‘drinking in the dark (DID),’ where exposure to 20% ethanol solution is

given two or four hours into the dark cycle (Rhodes et al, 2005). This was best demonstrated in C57BL/6J mice, an inbred strain with high preference for ethanol (Rhodes et al, 2005). However, DID fails to produce ethanol dependence and places constraint on ethanol access.

The 'alcohol deprivation effect' or ADE, was a notable paradigm because it incorporated several methods of alcohol administration that have been mentioned here, but for the purpose of producing an intense withdrawal episode. This was first demonstrated by Sinclair and Senter, by giving animals alcohol access for a particular period of time followed by a deprivation period that could be days, weeks or even months long (1967). Animals were given an alcohol access period during which a transient increase in ethanol intake could be observed, often characterized as a "relapse-like" phase. The duration of the alcohol access and deprivation period greatly influenced the ADE, and much variability was found among different strains of mice and rats (Vengeliene et al, 2014).

Intermittent Access to Ethanol.

Each model of ethanol drinking is an attempt to translationally model alcohol addiction, but no single model can fully capture all components of chronic human drinking behavior. An ideal model should have animals orally ingest alcohol voluntarily, prefer alcohol to other liquids, and demonstrate physical dependence to alcohol through display of withdrawal behavior (Lester and Freed, 1973; Dole et al, 1985).

The intermittent access protocol has been a promising translational model for chronic alcohol dependence. It has developed from observations that human drinking has

a particular pattern of heavy "binge" drinking periods interspersed with abstinence periods (Breese et al, 2005). This alternation of drinking with deprivation causes a "kindling" effect, where each subsequent episode of drinking that sensitizes the effects of ethanol withdrawal, increasing alcohol intake during the next drinking period (Ballenger and Post, 1978; Goddard et al, 1969; Heyser et al, 1997). The intermittent access procedure both replicates this cycle of drinking behavior and results in escalated drinking over time (Hwa et al, 2011). Wise first demonstrated in male Wistar rats how intermittent access produced persistent, high levels of 20% ethanol intake and sustained the effects of drinking longest, compared to continuous access (Wise, 1973). Intermittent access also escalated drinking of 20% ethanol solution in ethanol-preferring Long Evans rats as well as drinking of 15% ethanol solution in B6 mice (Simms et al, 2008; Melendez, 2011). Hwa and colleagues adapted the intermittent access paradigm by giving B6 mice 20% ethanol solution on Mondays, Wednesdays and Fridays, and demonstrated that animals on intermittent access drank significantly more ethanol over four weeks compared to those on continuous access (Hwa et al, 2011). The incorporation of deprivation days and voluntary access to orally consuming 20% ethanol solution makes this an ideal model for alcohol drinking in the current study.

Ethanol Withdrawal

Neurobiology of Relapse.

For chronic alcohol abusers, many symptoms may be present at withdrawal, including sweating, nausea, anxiety, disorientation, altered sleep pattern and seizures (Hasin, 2007; Mainerova, 2013). Alcohol withdrawal induced seizures are particularly

notable as a potentially fatal result of deprivation, and affect 10% of the alcohol abusers (Brown et al, 1988; Victor and Adams, 1953). Relief and avoidance of ethanol withdrawal symptoms have been considered motivating components of continued drinking (Gass and Olive, 2008; Roberts et al, 2000; Spanagel, 2009). The severity of withdrawal symptoms, including seizures, may increase with repeated episodes of heavy drinking and withdrawal (Goddard et al, 1969; Wada and Osawa, 1976; Pinel, 1980). Previously experiencing withdrawal seizures has been considered a predictor of increased severity of withdrawal symptoms in the future. In one study, 72% of alcohol abusers experiencing seizures had previously experienced alcohol withdrawal seizures (Brown et al, 1988). In animal studies, chronic ethanol exposure increases rate of increased seizure susceptibility and withdrawal severity (Pinel et al, 1975; Pinel and Van Oot, 1975; Pinel, 1980; Carrington et al, 1984; Walker and Zornetzer, 1974; Poldrugo and Snead, 1984).

The glumatergic system is believed to play a role in both alcohol dependence as well as these withdrawal symptoms (Tsai and Coyle, 1998). Glutamate is the most common excitatory neurotransmitter of the central nervous system, and has been studied in anxiety, depression and drug abuse (Lovinger, 2008; Spanagel, 2009; Conn & Pinn, 1997; Skolnick et al, 2009; Coyle, 2006). Glutamate can bind to three different ionotropic receptors, including the N-methyl-D-aspartate (NMDA) receptor; it is suggested that changes in glumatergic activity in chronic alcohol use are due to sensitization of the NMDA receptor increasing its receptive function (Spanagel, 2009; Tsai and Coyle, 1998; Gass and Olive, 2008). Various studies suggest that there is a neuroadaptive response to ethanol intake and dependence in regards to varying levels of glutamate due to chronic alcohol consumption (Moran et al, 2005; Idrus, 2011; Gass & Olive, 2008). Microdialysis

studies have demonstrated how chronic ethanol exposure over four weeks has elevated glutamate levels, while at six weeks the opposite effect is seen, suggesting that initial withdrawal creates hyperexcitability of NMDA receptors, while sustained deprivation reduces function (Gass and Olive, 2008).

Corticotropin Releasing Factor.

Corticotropin releasing factor, or CRF, has long been implicated with stress and anxiety-like behavior (Dunn and Berridge, 1990). CRF is a peptide hormone synthesized by a subset of neurons of the paraventricular nucleus of the hypothalamus, and functions in the stimulation of adrenocorticotropic hormone, ACTH, synthesis and release (Vale et al, 1981; 1983). It is also involved in the stress response of the HPA axis, as well as many other extrahypothalamic sites (Vale et al, 1981; 1983). Extensive research has focused on CRF 1 receptor antagonists that mediate several affects of alcohol dependence, such as anxiety and emotional state, by reducing ethanol consumption and relapse due to stress (Breese et al, 2011; Helig et al, 2010; Koob and Zorrilla, 2010; Zorrilla et al, 2012; Le and Shaham, 2002; Logrip et al, 2011). CRF 1 receptor antagonists have been successful in both blunting anxiogenic behavior as well as inducing anxiolytic behavior in animal models of anxiety, including defensive burying, conditioned fear, and ultrasonic vocalizations (Heinrichs et al, 2002; Zhao et al, 2007; Valdez et al, 2003; Griebel et al, 2002; Kehne et al, 2000; Shekhar et al, 2011). One potential caveat of mediating stress through CRF 1 receptor antagonists however, is that they are generally successful for high anxiety situations, but not low anxiety (Zorrilla and Koob, 2004).

CRF is a compelling component through which to study alcohol addiction and relapse because the CRF system is thought to be involved not only with the rewarding and reinforcing process of alcohol consumption and dependence, but also to the negative episodes of withdrawal associated with anxiety and stress (Pohorecky, 1991; Solomon and Corbit, 1974). Studies with CRF 1 receptor antagonists have noted reduced drinking in rats with high anxiety-like behavior, as well as reduction in anxiety-like behavior and withdrawal symptoms when CRF antagonists are inject into areas of the central amygdala (Parylack et al, 2011; Koob and Zorrilla, 2010; Logrip et al, 2011; Ciccocioppo et al, 2006; Hansson et al, 2006; Hanson et al, 2007; Heilig and Koob, 1997; Lodge and Lawrence, 2003; Sommer et al, 2008). CRF 1 levels are also notably altered post-repeated ethanol administration/withdrawal cycle, further implicating the CRF system in dependence and withdrawal (Sommer et al, 2008; Zorrilla et al, 2001).

Seizures.

Withdrawal symptoms, including seizures, have been shown to decrease in severity and incidence with NMDA antagonists, while administration of NMDA in ethanol dependent animals has increased handling induced seizures (Grant et al, 1990; Biekkowski et al, 2001). For rats in ethanol withdrawal, NMDA antagonist dextromethorphan decreased audiogenic seizures, with similar results in mice with antagonists MK-801 and MRZ 2/579 (Erden et al, 1999; Bienkowski et al, 2001). Acamprosate, an NMDA receptor co-agonist which has been used as an approved drug treatment for alcoholics, has also attenuated anxiety-like behavior in ethanol withdrawn rats (Kotlinska & Bochenski, 2008).

Seizures that occur human alcohol drinkers in withdrawal have been modeled in both mice and rats through handling induced convulsions (Mello, 1972; Goldstein, 1972). In continuous chronic alcohol dependence, convulsions can be seen after 8 weeks of drinking (Ozburn et al, 2013). Though handling induced convulsions have been a useful tool in studying and determining whether a subject is experiencing ethanol withdrawal, it is only present late into dependence in animal models. Many other studies have looked at other affective withdrawal signs, such as anxiety-like behavior, to characterize withdrawal as well (File, 1994; File and Andrews, 1991; File et al, 1993; Gatch et al, 1999; Overstreet et al, 2002; Valdez et al, 2002). By shifting focus to anxiety-like behavior as a component of withdrawal, it may be found that like, seizures, disruptions in such behavior may predict drinking intake outcomes as well as withdrawal severity.

Anxiety

Anxiety, one of the common symptoms associated with withdrawal in human alcohol abusers, has been studied in several capacities. It is described as a response to perceived threat to well-being, and includes defensive behaviors, arousal, and neuroendocrine activation (Steimer, 2002; Steimer, 2011). In alcohol-induced withdrawal, relapse is often associated with cues and stress-induced cravings, including anxiety (Breese et al, 2005; Cooney et al, 1997; Tsai et al, 1995). Motivation for increased alcohol consumption has indeed been associated with increased anxiety, and cues associated with alcohol during abstinence are also found induce anxiety, alcohol craving and increased physical stress response (Kushner, 1994; Overstreet et al, 2002; Chiang et al, 2002; McCusker and Brown, 1991; Fox 2007).

Both conditioned fear responses, sustained fear, also known as anxiety, and phasic fear, have been associated with fear circuitry in the amygdala (Gray and Magnuson, 1987, 1992; Holstege et al., 1985; Hopkins and Holstege, 1978; Peyron et al., 1998; Rosen et al., 1991; Schmued, 1994; Schwaber et al., 1982). A number of startle studies, including fear potentiated startle, light potentiated startle and CRF potentiated startle have indicated that phasic and sustained fear are categorically different (Blanchard et al., 2003; Blanchard et al., 1993, Walker, 2009). Additional lesion and inactivation studies have further indicated that each are regulated by different neural systems (Sakanaka et al., 1986; Koob et al., 2004; Goosens and Maren, 2001; Zimmerman et al., 2007; Davis and Whalen, 2001; Jasnow et al., 2004, Erb et al., 2001). Glutamatergic release has been particularly implicated in sustained fear associated behaviors; rats receiving chronic stress had increased glutamate levels (Santibanez et al, 2005). Brain regions and circuitry associated with sustained fear have been pharmacologically manipulated to block the effects of withdrawal-induced place aversions (Cecchi et al, 2007; Forray et al, 1995; Forray et al, 1997).

Modeling Anxiety in Animals.

Anxiety-like behavior has been difficult to translate comprehensively in animal models. Conditioned test models involve an associative learning process in order to induce an affective response (Ventura-Silva et al, 2013; Koolhas et al, 2011). They vary from conflict tests, such as the Vogel Drinking and the Geller-Seifter tests, to avoidance tasks, like passive avoidance and fear-potentiated behavior (Howard and Pollard, 1991; File, 2004). These tests, while useful for screening anxiolytic compounds, require much training and reduce the translational quality of the motivation involved in anxiety-like

behaviors (Steimer, 2011; File, 2004). Ethological models that do not involve conditioning attempt to replicate natural threat in order to invoke responses of fear or curiosity (Ventura-Silva et al, 2013). Montgomery was the first to create this type of task using a Y shaped field, in order to observe exploratory behavior in rats (1955). Other models of this type include the elevated plus maze, light/dark box, and social interaction. While these tests are more translational in modelling anxiety like behavior, they cannot often be repeated, and rely on the inference that changes in a certain type of behavior is indicative of a change in anxiety like behavior. No single model has been able to achieve the full range of anxiety in totality; instead, each model has particular advantages that allow for study of certain component of anxiety like behavior. For a review of animal models of anxiety-like behavior, refer to Table 1.

Unconditioned Response Models					
Model	*Type of Test*	*Measure*	*Advantages*	*Disadvantages*	*Reference*
Open Field	Exploratory	Observation of animal in exploring unknown environment	Can be used across a variety of species; simple; can be used to screen many kinds of drugs-particularly robust for anxiolytic benzodiazepines	Has low predictive validity; many different behaviors observed, indicative of more than just anxiety	Hall, 1934; Walsh and Cummins, 1976
Elevated Plus Maze	Exploratory	Duration of time spent in aversively associated open arms versus closes arms	Used in several animal models; highly reliable; exploratory translational model; light independent	Not as robust for non-benzodiazepine anxiolytics; issue of avoidance or escape	Pellow et al, 1985; Rodgers et al, 1995; Cruz et al, 1994
Dark/ Light Box	Dark/Light transition	Track transitions moving between light	Highly translational; exploratory behavior of novel environment;	Cannot be repeated; requires prior habituation to	Crawley 1981; Costall et al, 1989;

		and dark fields	locomotor activity	consistent light/dark cycle; hard to distinguish between changes in activity; strain differences	
Ultra Sonic Vocal-izations	Social	Patterns of US vocalizations by pups and/or adults indicative of interaction or response to danger	Can be accompanied by defensive behavior; robust in screening benzodiazepine anxiolytics	Not as robust for other types of anxiolytic compounds; modulation due stress/anxiety is relatively unknown	Scattoni et al, 2009; Crawley, 2012; Sanchez, 2003
Social Interaction	Social	Decreased time spent with stimulus animal indicative of increased anxiety	Natural social behavior; works for studying benzodiazepines and other anxiolytics; locomotor behavior; no training	Implications of repeat testing not well known; relies on duration as sign of anxiety	File and Hyde, 1978; File, 1980; File and Seth, 2003; Moy, 2004
Conditioned Response Models					
Model	*Type of Test*	*Measure*	*Advantages*	*Disadvantages*	*Reference*
Geller-Seifter	Conflict	Pressing for reward despite punishment presentation	Test can be repeated over long period of time	Requires training; operates on schedule	Geller and Seifter, 1960; Charrier et al, 1994
Vogel Drinking	Conflict	Water deprived animals shocked for drinking water	Robust in screening anxiolytics; no training required, efficient to test; useful for testing across strains/species	Deprivation required; drug manipulation alters impact of punishment/may interfere with drinking	Vogel et al, 1971; Lippa et al, 1977
Defensive Burying	Avoidance	Defensive burying behavior suppression indicative of anxiety like behavior	No pre-training required; useful for studying novel anxiolytic compounds	Shock may mask anxiolytic effect in some conditions	Treit et al, 1981; Treit et al, 1991
Fear Potentiated Startle	Avoidance	Stimulus associated with aversive foot shock; stimulus then presented with noise causing startle, potentiating	Within-subjects comparison with and without stimulus; no obvious operant; training and testing sessions are separate	Not as sensitive as other conditioned response models	Chi, 1965; Davis, 1979; Appel 1963; Millenson and Leslie, 1974

		effect			
Conditioned Taste Aversion	Avoidance	Aversive stimulus paired with novel taste	May be indicative of natural defense mechanism	Unclear if effect from anxiolytics is due to dipsogenic effects or anti anxiety; in drug studies, drug itself may have taste aversion	Garcia and Koelling, 1966; Concannon and Freda, 1980; Cooper and Francis, 1979

Table 1: Animal Models of Anxiety
The following table describes several commonly used behavioral models of anxiety. The name of the test is given, followed by the type of model. The measure is the behavior that is being altered in the test to indicate change in state of anxiety-like behavior, followed by the several advantages/disadvantages of each test. Original references are provided at the end.

Social Interaction.

Social attraction was observed as a natural phenomenon first by Latane and Glass in 1969. The social interaction (SI) test was formally developed by File and Hyde based on this behavior, and was one of the first reliable ethological models of anxiety like behavior in animals (1978). In this model, training, electric shock and deprivation, all components of previously used conditional response anxiety models, were avoided, instead focusing on the natural sociability of the animals. The protocol required a pair of two male rats, treated as a single unit that was observed and scored for duration of time spent interacting with one another in an open field. The use of a novel, neutral location diminished territorial aggression by the subject (Kuti and Page, 2011). This protocol did not hinder overall locomotor activity. Increased social interaction is considered to be indicative of anxiolytic behavior, and decreased social interaction is indicative of anxiogenic behavior; the ability to see effects in both directions is unique to the SI test (File and Seth, 2003). The social interaction test was validated as a behavioral measure

for anxiety-like behavior in rodents because of its success in demonstrating anxiolytic effects of clinically used anxiolytic compounds (File and Seth, 2003; Kuti and Page, 2011). This feature also made it ideal for screening novel anxiolytic compounds as well as other manipulations that were non-pharmacological.

Though originally developed for male rat pairs, the model has also been useful for both gerbils and mice (de Angelis and File, 1979; Lister and Hikakivi, 1988; Krsiak et al, 1984; Krsiak and Sulcova, 1990). In these animals, changes in social interactions could be observed, though to different extents when compared to rats. Additionally, particular strains of mice were found to vary in reliability to the SI test. Outbred strains such as Swiss Webster were more reliably social, though had instances where they had to be separated into subgroups in order to see effect; inbred strains on the other hand, such as B6, performed less consistently in the SI test, and another inbred strain A/J, essentially demonstrated no social activity (Olivier and Mos, 1988; Moy 2004). In these mouse models, the original SI protocol was altered: subject animals were observed in relation to contained stimulus animals in one chamber, as opposed to an empty chamber, removing the need to lump each pair as a single unit (Moy, 2004). The choice component of the social interaction test allows protocols to look specifically to look at interaction behavior and not investigation by the mouse (Kuti and Page, 2011). In earlier protocols of social interaction, subjects and stimulus animals were matched for sex to minimize possibility mating-like behavior; more recently, ovariechtomized and orchidectomized stimulus animals have been used to further remove the possibility of sexually motivated behavior (Kuti and Page, 2011). For female stimulus animals, differences in estrous cycle at the time of the test is also removed as a potentially confounding factor.

Pharmacological studies have linked CRF to anxiety like behavior responses. CRF injected inrtacerebroventricularly has been found to have an anxiogenic effect, which can be blocked by fluoxetine (Dunn and File, 1987; To et al, 1999). Social interaction has been found to increase in male rats post combined CRF and arginine vasopressin infusions (Elkabir et al, 1990). Peptide urocortin, known to have CRF receptor affinity, has been found to produce an anxiogenic effect, while CRF receptor antagonist astressin injected in the basolateral amygdala can reverse the urocortin effect (Sajdyk et al., 1999; Sajdyk and Gehlert, 2000).

Social Interaction and Ethanol.

Dose effect curves have been observed from studies that compare the effects that different doses of alcohol administration have on social interaction. In both adolescent and adult rats, low doses of ethanol demonstrated anxiolytic effects, while higher doses produced poor motor control (File, 1980; File et al, 1976). Furthermore, increased social interaction and social preference were noted at low doses of ethanol while higher doses saw decreased social interaction and avoidance (Varlinkskaya et al, 2001).

The SI test has also been useful in studying the anxiogenic effects due to withdrawal from drugs of abuse, such as benzodiazepines, nicotine and ethanol (File et al, 1998; Irvine et al., 1999; Irvine et al., 2001; Vellucci and File, 1979; Fernandes et al, 1999; Baldwin and File, 1989; Baldwin et al, 1990; File et al, 1991; Andrews and File, 1992). Various rat strains have been observed to show increases in anxiety like behavior in the SI test following deprivation from ethanol (Kampov-Polevoy et al, 2000; File et al, 1989, 1992). Several deprivation episodes from ethanol has been found to increase the

anxiogenic effect of withdrawal; in deprivation from ethanol diet, anxiogenic behavior was also exhibited, the effects of which have been reversed by flumazenil, chlordiazepoxide and baclofen (File et al, 1989; File et al, 1992; Overstreet et al, 2002).

Objective

Many studies have studied the onset of dependent ethanol consumption, as well as the maintenance of drinking, particularly due to the effects of withdrawal that contribute to relapse of drinking. In animal models, while ethanol dependence and withdrawal can be characterized by handling induced convulsions, such behavior is only apparent in late chronic consumption. This study aims to focus on another effect induced by ethanol withdrawal, anxiety, in order to study the more sensitive behavioral changes that may be taking place in the transition to dependent ethanol consumption. By utilizing a social interaction test at various time points as drinking intake escalates, it is predicted that increased anxiety-like behavior will be seen over time in ethanol-withdrawn mice. It is further hypothesized that such early changes in social interaction in these mice may have predictive value when compared to induced seizure activity later on. With no change in locomotor activity over time, these changes may be indicative of nuanced effect of ethanol withdrawal due to increased anxiety-like behavior.

Methods

Animals.

Adult male and female Swiss Webster mice (Carworth Farm Webster, CFW from Charles River Laboratories, Rhode Island). On arrival, mice weighed 23-25 grams and were group housed by sex in polycarbonate cages (28 x 17 x 12 cm) with pine shavings

(Shepherd's Specialty Blend Alpha-dri) bedding. Mice were given approximately one week for habituation to the vivarium, which was kept on a reverse 12 hour light-dark cycle (dark hours were 600 to 1800). The vivarium was maintained at 21 +/- 1°C with 20% humidity. Stimulus subjects, see below, both male and female mice, were given orchidectomy or ovariectomy surgeries, and were post-operatively monitored for a week in single housing conditions. Upon successful healing of incision and wound clip removal, stimulus animals were subsequently group housed by sex. Another group of male CFW mice served as the experimental subjects (n=36), and were single-housed post habituation in the same cage conditions, including a wire stainless steel lids with two openings for inserting nozzles of drinking bottles. Behavioral testing for these animals occurred during the dark cycle phase, and all bedding was changed at least 48 hours prior to testing. For both stimulus and experimental animals, Purina 5001 Rodent chow and tap water were available ad libitum. Animal care and use procedures were all approved by the Tufts University Institutional Animal Care and Use Committee, implementing the NIH Guide for Care and Use of Laboratory Animals (2011).

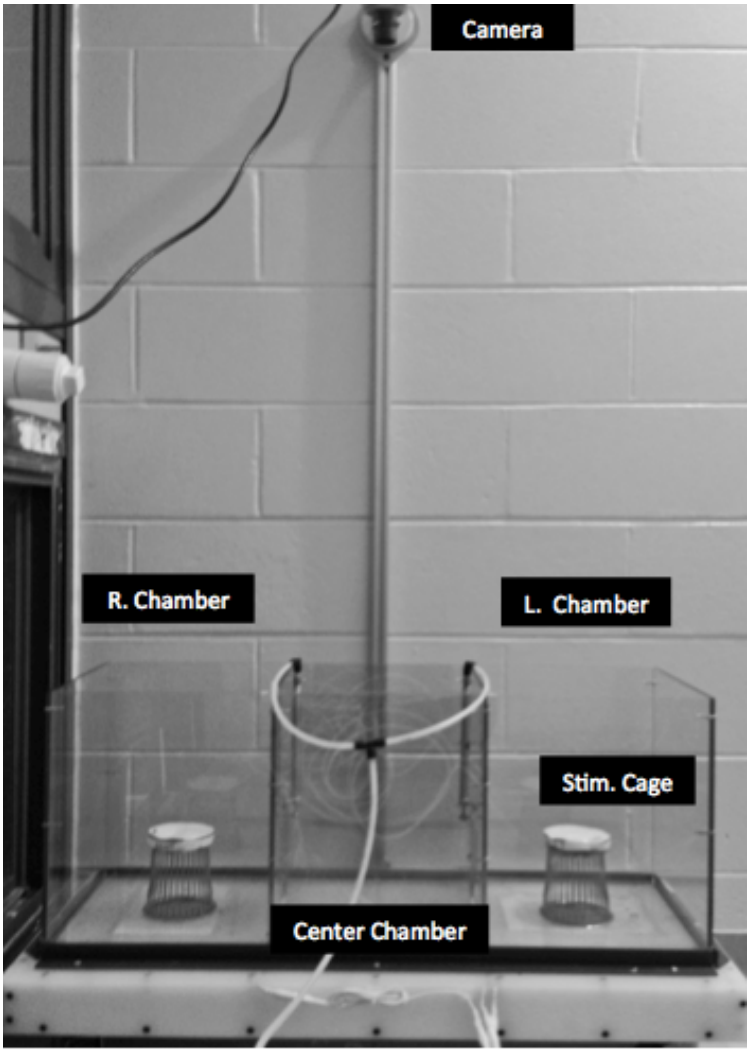
Materials.

Social Interaction Apparatus.

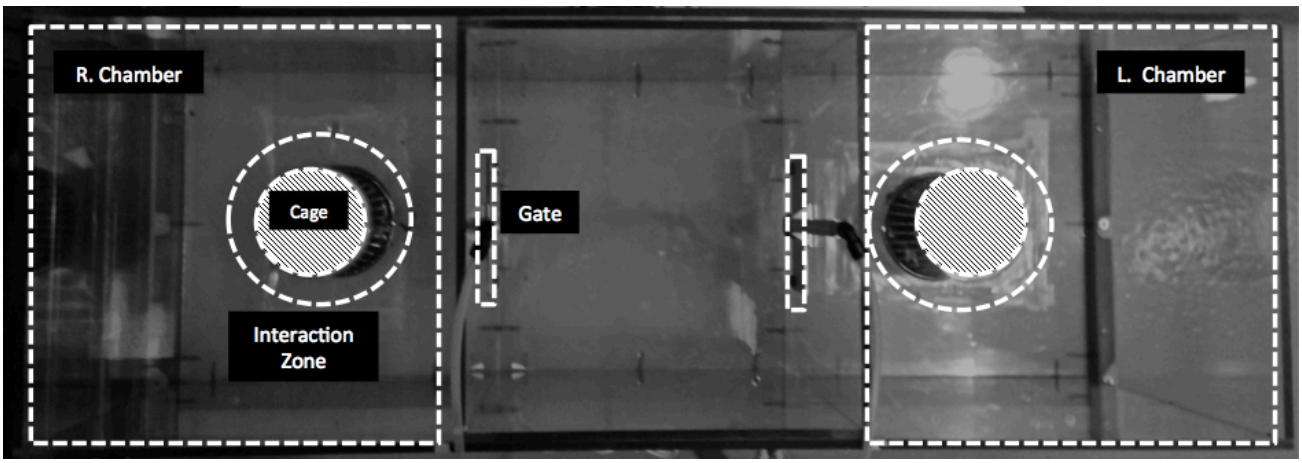
A three-chambered apparatus was introduced as an effective tool for studying social interaction and social preference in rodents by Moy (2004). The rectangular apparatus used in the present investigation was comprised of three equally sized square chambers (29 x 29 x 36 cm), all cut from transparent polycarbonate. Removable floor panels were fitted to ensure consistent placement of the two wire stimulus mouse cages within the left and right chambers. To prevent any movement during the experimental

session, a circular weighted disk was fitted and place atop each cage. The apparatus included two, removable center chambers to allow for concurrent habituation and behavioral testing. The doors, located on either side of each center chamber, were opened and closed remotely to prevent interference with the automated tracking system, see below. During the five minute habituation period, the two doors remained closed by maintaining pressure within the two air pistons installed above each door (Central Neumatic). To initiate the testing session, pistons were depressurized remotely and the doors were opened, allowing the experimental mouse to freely roam the entire three chamber apparatus. The floor of the apparatus housed red LED lights for tracking. For detailed schematic of apparatus, refer for Figure 1:

A)


Camera
R. Chamber
L. Chamber
Stim. Cage
Center Chamber

B)


R. Chamber
Cage
Interaction Zone
Gate
L. Chamber

C)

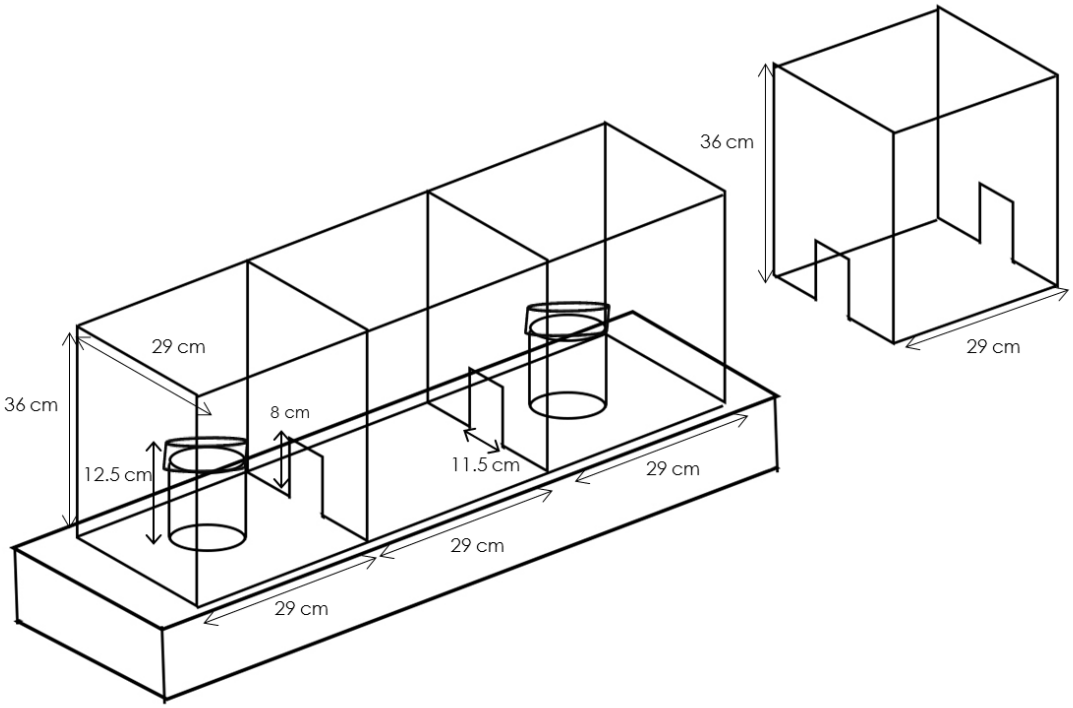


Figure 1: Social Interaction Apparatus

The above figures describe the social interaction apparatus used for the present study. Figure A is photographic front view of the entire apparatus set-up, with the three chambers labeled, and the camera securely positioned 173 cm above the chamber. The additional center chamber is not shown here. Figure B is also a photographic image of an overhead view of the apparatus, with the interaction zones comprising 3.8 cm from the outside of the stimulus cages. The removable second center chamber is also not shown here. Figure C is an overall scheme of the three chamber apparatus as well as the second removable center chamber.

Behavioral Measurements.

For the social interaction test, the tracking sessions were recorded with the Panasonic WV-CP280 or the JVC Everio GZ-MG670 digital camera, which was securely mounted to a stand attached to the center of the back outer wall of the apparatus. The

camera was mounted 173 cm above the apparatus. Tracking was done with *The EthoVision XT* software (Noldus,11.5; Wageningen, The Netherlands), and recorded frequency of entry, latency to enter, and duration of time spent in the left and right chamber, and left and right sniffing zones, as well as total distance traveled. The tracking points included a head point, tail point and center body point. The interaction zone was a 3.8 cm radius from the edge of the stimulus cage, which was calibrated for every testing session.

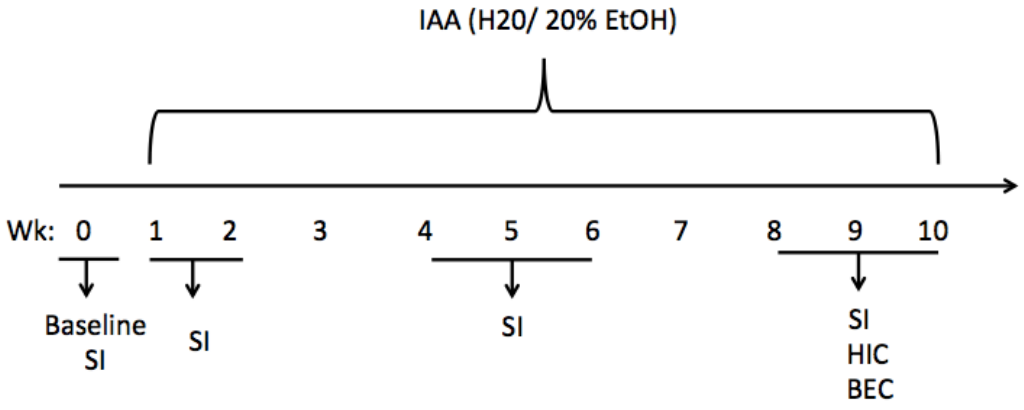
Ethanol Intake Procedures.

Four days prior to baseline testing for social interaction, experimental subjects were presented with two 50 mL Nalgene centrifuge tubes with stainless steel ball bearing sippers (Ancare Corp., Bellmore, NY) on number 5 rubber stoppers (Fisher Scientific, Agawam, MA). The tubes contained tap water, and were used to acclimate the subjects drinking from the sipper nozzles. 20% weight/volume ethanol solution was prepared weekly with tap water and 95% ethyl alcohol (Pharmaco-AAPER, Brookfield, CT). Tubes were placed in the openings in the wire mesh lids approximately 3 hours into the dark cycle (900 hours) daily, and weights of bottles were recorded 24 hours later after placement. Weights were recorded to the nearest hundredth of a gram and converted to grams of fluid consumed per kilogram of animal body weight. Due to the possibility of accidental spillage or evaporation, a drip cage was prepared without an experimental animal. Weekly drip averages of fluid lost were calculated and subtracted from daily ethanol intake. Water control animals were given only water, the intake of which was not recorded. Both water controls and experimental subjects were weighed every other day to the nearest tenth of a gram.

Intermittent Access to Ethanol.

Experimental subjects had intermittent access (IAA) to 20% ethanol solution as described by Hwa et al (2011). Each day, experimental mice had access to two bottles. On Tuesdays, Thursdays and Saturdays, mice were presented with one bottle containing the 20% ethanol solution, and the other bottle containing tap water; both bottle weights were recorded. Mice had access to the bottles for 24 hours, and on Wednesdays, Fridays, Sundays and Mondays, the ethanol bottle was weighed and then thoroughly cleaned and replaced with tap water. For every ethanol access day, the position of the bottle was alternated to either the left or side of the wire cage in order to avoid potential side preferences.

A)



B)

Sun	Mon	Tue	Wed	Thur	Fri	Sat
Water/ Water	Water/ Water	**EtOH/ Water**	Water/ Water	**EtOH/ Water**	Water/ Water	**EtOH/ Water**
SI Test 2			SI Test 2			

Figure 2: Experimental Design

Figure A is the overall experimental design of the present study. Baseline social interaction measures were taken at Week 0, followed by the IAA protocol, which spanned from weeks 1-10. Social interaction testing took place at Weeks 1-2, 4-6, and 8-10. Post social interaction testing at Week 8-10, HIC scores were performed and BEC samples were collected and analyzed. Figure B portrays a typical weekly schedule: ethanol access days were Tuesday, Thursday and Saturday; the remainder were water/withdrawal days. Social interaction testing would take place on the first and last withdrawal day, no testing took place on the second day.

Social Interaction Test.

The social interaction test used in this experiment was modified from the protocol described by Moy (2004). Testing sessions for social interaction took place a week before intermittent access to ethanol began (baseline), week 1 of IAA, week 4 of IAA and week 9 of IAA. Experimental animals had ethanol access taken away 3 hours into dark cycle (900 hours), and given water instead. Control water drinking animals were tested for social preference at 1200 hours, and experimental animals were tested 6-8 hours after bottle removal (1500 hours). Only one testing session was completed a day, and female and male stimulus mice were alternated every testing session; no more than two testing sessions took place in a single week.

The tracking software allowed for the designation of the stimulus wire cages as "cage zones," while the area immediately outside the cage zone was designated as the "sniffing zone;" the arena and track settings were calibrated using the camera feed prior to each testing session. The testing session began with the experimental animal being placed in the central chamber of the apparatus with the gates fully closed. The

experimental animal was given 5 minutes to habituate to the chamber, after which time a stimulus animal was placed in either the left or the right chamber in the cylindrical wire mesh cage, while the other cage remained empty. From outside the test room, the researcher could release a pressure switch to automatically open the center chamber gates, allowing the experimental animal to freely move throughout the whole apparatus for 10 minutes. The tracking software measured frequency of entry, latency to enter, and duration of time spent in the left and right chamber, and left and right sniffing zones, as well as total distance traveled. The tracking points included a head point, tail point and center body point, all of which were included in analyses. After the trial with the experimental subject was completed, the stimulus animal was marked to ensure that no stimulus animals were repeated in the test for the remainder of the session. The apparatus was thoroughly cleaned with Vimoba solution, as the next experimental animal habituated in the removed clean center chamber, which was then inserted into the clean apparatus.

Handling Induced Convulsions.

After the final 8-10 week range social preference test was completed, experimental subjects were assessed for severity of ethanol withdrawal at the next ethanol drinking day, through handling induced convulsions (HIC) as described by Goldstein (1972). Both water and intermittent access ethanol drinking mice were lifted by the tail and observed in order to determine their HIC score through this 0-4 scale: 0 = no withdrawal signs; 1 = tonic convulsions induced by gently turning the mouse 180º; 2 = tonic convulsions when lifted without turning or tonic-clonic convulsions when turned 180º; 3 = tonic-clonic convulsions without turning the mouse; 4 = violent tonic-clonic

convulsions when lifted that often continue when mouse is placed back in cage. HIC scores were taken both 4 and 8 hours into withdrawal, after ethanol bottle was removed and replaced with bottle for IAA experimental subject mice.

Blood Ethanol Concentrations.

Blood samples were taken from IAA experimental subjects after at least 8 weeks of intermittent access to ethanol, and at least 24 hours after HIC scores and 48 hours after last social preference test. Mice had been given 1 hour access to one bottle of 20% ethanol and 1 bottle of tap water, after which bottles were removed and weighed to determine intake for both fluids. Blood samples were then taken from each subject from the submandibular vein, and immediately centrifuged at 21°C for 10 minutes at 3,000 rpm in order to distill plasma. Plasma samples were analyzed for blood ethanol concentration (BEC, mg/dl) using AM1 Analox (Analox Instruments Ltd, London, UK). The analyser was calibrated using a 100 mg/dl ethanol standard sample before each batch of plasma samples, and an alcohol oxidizing reagent was used to determine the BEC content of each sample (GMRD 113, Analox Instruments Ltd).

Statistical Analysis.

All statistical analyses were completed with SigmaPlot 11.0 (Systat Software, San Jose, CA). For both ethanol intake (g/kg) and ethanol preference (%) between High and Low Drinking groups, two way repeated measures ANOVAs were performed to determine main effects over time and between groups, and were supplemented with post-hoc Bonferroni t-tests to determine significance ($p<0.05$). In order to separate subjects

into the High and Low Drinking groups, a set of criteria was established: consistent consumption of 15 g/kg or higher every ethanol access day for the last four weeks of IAA for the High Drinking group (n=6); consistent consumption of 10 g/kg or less every ethanol access day for the last four weeks of IAA for the Low Drinking group (n=5), an intake that would be too low to induce any significant intoxication in these animals. These groups were found to be statistically different with a two-way repeated measures ANOVA in order to compared for the remaining tests for social interaction, BEC and HIC scores. All remaining subjects fell into an On/Off group due to their inconsistent drinking patterns; this group was omitted from statistical analyses involving BEC's and HIC scores.

Group separation was necessary to determine the effect of repeated withdrawal over time in the social interaction test. Additionally, both groups were compared against a control group (n=12). Duration of the time spent in the interaction zone (s) was compared among all three groups and over four time points of IAA (Week 0, 1, 4, 8) using a two way repeated measures ANOVA for social interaction with a female stimulus and for social interaction with a male stimulus. Post-hoc Bonferroni t-tests were used to evaluate specific comparisons within significant effects. A two-repeated measures ANOVA was also used to determine effects of time and group for percent change from baseline, which was Week 0 for each stimulus type. Locomotor activity was also assessed for effects across group conditions and over time with two way repeated measures ANOVAs, and significance was determined with post-hoc Bonferroni t-tests.

Blood ethanol concentration (BEC) levels were analyzed with the Analox analyzer and calibrated to a 100 mg/dl ethanol standard. BEC levels were grouped by

High and Low Drinking groups and averaged; a one way ANOVA was performed to determine group effect and significance confirmed with a post-hoc Bonferroni t-test. HIC scores were analyzed for effects of group and hours into withdrawal (4 and 8) and were reported nonparametrically in median and interquartile ranges.

Results

Ethanol Intake and Preference.

Consumption of 20% ethanol was assessed over 8 weeks in adult CFW male mice (n=24), following the intermittent access procedure. Intake per ethanol drinking day was recorded; variability in total career consumption (Figure 3) and daily intake allowed for the separation of subjects in a High drinking group and Low drinking group (Figure 4). The specific criterion for the High drinking group was daily consistent consumption of at least 15 g/kg for the last four weeks; criterion for the Low drinking group was less than 10 g/kg daily for the last four weeks. The mean intake for the High drinking group was 18.8±0.9 (g/kg) and the mean intake for the Low drinking group was 6.2±1.1 (g/kg). A two-way repeated analysis of variance (ANOVA) revealed a significant main effect of group type [$F(1, 207)=73.693$, $p<0.001$], and a post-hoc Bonferroni t-test confirmed that the High drinking group drank significantly higher than the Low drinking group for twenty-one of the twenty-four ethanol days ($t=8.584$, $p<0.001$). A second main effect was found over time [$F(23, 207)=2.229$, $p<0.002$] for drinking days 13 and 14 in the High drinking group, where a post-hoc Bonferroni t-test revealed higher intake than the first drinking day for both of those day ($t=4.345$; $t=3.850$; $t=3.857$, $p<0.050$). No significant difference was found over time for the Low drinking group, and both High

and Low drinking groups remained relatively stable for the last four weeks of intermittent access. Career consumption ranged from 64.23 to 666.11 g/kg.

Preference for ethanol was also assessed for each of the groups over the 8 week IAA protocol. Preference was calculated by dividing ethanol volume drank (ml) by the total fluid (ethanol and water) (ml) (Figure 5). A significant effect was found with a two-way repeated measures ANOVA performed between the drinking groups [$F(1, 207)=30.091$, $p<0.001$], with a post hoc Bonferroni t-test confirming that the High group showing a significantly higher preference for ethanol than the Low drinking group ($t=5.486$, $p<0.001$). The average preference for the High Drinking group was 0.48±0.04, and the average preference for the Low Drinking group was found to be 0.14±0.05; preference remained lower than 50% for the Low Drinking group for all 8 weeks; for 11 drinking days, the High Drinking achieved a preference of 50% or higher.

Social Interaction: Time Spent in Interaction Zone and Percent Change in Baseline.

Social interaction with the stimulus animal (male, female) was recorded over time (Weeks 0, 1, 4, 8) and measured in duration of time spent in the interaction zone (s). No main effect or interaction between group or over time was found for the male stimulus test according to a two-way repeated measures ANOVA. However the two-way repeated measures ANOVA found a trend-like effect of time for the female stimulus test [$F(3, 59)=3.641$, $p=0.071$]; a post-hoc Bonferroni t-test revealed that Week 0 was significantly lower in time spent in the interaction zone than Week 0 for the Low Drinking group ($t=3.210$, $p<0.05$). From the interaction duration times, percent change from baseline (which

was Week 0) for each week (Week 1, 4, 8) was determined, but no significant interaction or effect was found across groups or time.

Locomotor activity was measured during the social interaction test session (cm) for both the male and female stimulus animal. A significant effect of time was found for all groups test [$F(3, 33)=17.444$, $<0<0.001$], confirmed by a post-hoc Bonferroni t-test ($t=6.945$, $p<0.001$; $t=5.223$, $p<0.001$; $t=3.962$, $p<0.01$; $t=2.983$, $p<0.05$) that saw an increase in motor movement at week 8 compared to weeks 0, 1, and 4 for the female stimulus condition. The same significant effect in time was found in the male stimulus condition test [$F(3, 54)=39.572$, $p<0.001$], with an increase in locomotor activity at 8 weeks compared to Weeks 0,1 and 4, confirmed by a post-hoc Bonferroni t-test ($t=9.392$, $p<0.001$; $t=9.356$, $p<0.001$; $t=4.5$, $p<0.001$).

Blood Ethanol Concentration.

Blood ethanol concentrations ranged from 4.3 to 153.8 mg/dl. Average for the Low Drinking group was 35.8±7.4, and average for the High Drinking group was 50.825±34.6. No significant difference was found between the two groups.

Handling Induced Convulsion (HIC).

HIC scores were assessed according to the rating scale described by Goldstein (1972) for 6 High Drinking mice, 5 Low drinking mice and 12 Control subject. HICs were performed 4 hours into withdrawal and 8 hours into withdrawal following 8 weeks of IAA to ethanol. A Kruskal-Wallis one-way ANOVA by ranks revealed a significant group effect for the 8 hour withdrawal [$H= 20.213$, $p <0.001$], but not for the 4 hour

withdrawal. A post-hoc Dunn's Method test confirmed that the High Drinking group and Low Drinking group both had significantly higher HIC scores than the Control group (Q=3.440, p>0.05; Q= 3.130, p<0.05). Median HIC scores were the same for the High and Low Drinking groups across 4 hour and 8 hour withdrawal.

Discussion

Ethanol Drinking and Preference.

Escalated drinking of 20% ethanol was found in the outbred CFW male mice on the intermittent access procedure, as described by Hwa et al in 2011 for inbred C57BL/6J mice. Overall, the CFW mice in this study had much variability both in daily intake and overall career consumption, which is characteristic of outbred strains and ethanol drinking (Kosobud et al, 1988; Allen et al, 1982; Chia et al, 2005). Separation into a High Drinking group and Low drinking was based on different average intake between the groups, as well as stable drinking patterns for the last four weeks of the intermittent access procedure; this categorization, while arguably arbitrary, has been utilized in previous studies with outbred CFW male mice and ethanol drinking during IAA (Nathanson, *unpublished*). Statistically, the two groups were significantly different for intake and preference. Mice that drank inconsistently high and/or low amounts of ethanol were placed in an On/Off category, that had no significant effect in intake over time, and had no other significant effect or patterns of behavior in any of the subsequent testing for social interaction, and excluded from further analyses for BEC levels and HIC scores.

The preference of the High Drinking and Low Drinking groups were found to be significantly different, and the High Drinking group was able to achieve at least 50% or

higher preference on a third of the IAA protocol. However, the inconsistency of achieving 50% or greater preference makes it difficult to conclude the overall extent to which the High Drinking group preferred ethanol, only that they significantly preferred it on average more than the Low Drinking group. The individual differences for each subject's preference for ethanol is characteristic of outbred CFW mice, as is the overall average preference of less than 50% for even the High drinking group. Ultimately, the High drinking group was able to drink a consistent average of 18.76 g/kg over 8 weeks, indicating that a voluntary ethanol drinking protocol can be successful in escalating drinking in outbred mice.

Withdrawal Severity.

The question of whether these subjects were truly dependent on ethanol is more difficult to answer. Besides escalated drinking, ethanol preference, consistently high intake, and high overall career consumption, the determination of dependence is largely based on the severity of withdrawal, which is thought to be indicative of physical dependence (Goddard et al, 1969; Wada and Osawa, 1976; Pinel, 1980). HIC scores late into chronic drinking activity have been associated with withdrawal from ethanol, and thus associated with drinking dependence (Goldstein and Paul, 1971; Metten and Crabbe, 2005; Crabbe et al, 1980). The HIC Scores in this study did differ significantly between the Control group and the High and Low Drinking groups when assessed 8 hours after withdrawal, but were not significantly correlated with social interaction duration at any time point. The lack of difference in HIC scores between the Low Drinking and High drinking group may not necessarily indicate that withdrawal symptoms are not present or that dependence has not been achieved. HIC scores are related to not only longevity of

drinking, but also the consumption prior to withdrawal from ethanol (Goldstein, 1972). Due to the variability in drinking behavior of these outbred mice, there might also be variability in presence or severity of convulsive activity. Many outbred strains, including CFWs, have been reported to have variable, more sensitive results for HIC scores, suggesting that the HIC scores seen might be a true effect of withdrawal, but that ones without HIC scores may still possibly be in withdrawal (Metten and Crabbe, 2005). The severity of withdrawal remains unclear, particularly as individual differences exist in the rate of drinking throughout the 24 hour access period. For this reason, it is difficult to conclusively say that all ethanol drinkers, particularly high drinkers, drink most in the first hour of access.

The blood samples used to determine the BEC levels encountered a similar limitation. Since there is no way to determine at what time point each subject drinks most, one hour consumption amounts (g/kg) prior to blood sampling required corresponding BEC levels to be considered as true indication of ethanol drinking. Generally, in CFW male mice, among other mouse strains, blood ethanol content is thought be highest one hour after ethanol exposure; however, the range of BEC levels, even for subjects in the High drinking group, does suggest individual differences are an important consideration. Additionally, blood samples were collected only after all social interaction testing was completed at week 8 of IAA, making it difficult to conclude what the state of ethanol dependence and intake may have been a various points over the entire 8 week protocol. Blood sampling taken from the submandibular vein may be stressful and affect social interaction testing and/or drinking behavior, which is why it was not done prior to each testing week in this study. Future studies looking at escalated drinking and

behavioral testing may benefit from less stressful or invasive ways to take blood samples in order to better assess extent to which alcohol is truly consumed individually, as well as over time.

For many of the studies that use social interaction to study alcohol deprivation, voluntary ethanol access is not used. Instead, ethanol vapor chambers or injections are often used to induce a highly intoxicated state and ensure a follow-up deprivation episode. Prior studies utilizing these models of ethanol access have found decreased social interaction and social preference during ethanol withdrawal (Cutler, 1976; Kampov-Polevoy et al, 2000; Overstreet et al, 2002). It may be that even the High Drinking outbred animals in intermittent access were not intoxicated enough to have a real withdrawal episode

Social Interaction and Locomotor Activity.

Contrary to the results of similar studies involving ethanol withdrawal and social interaction, the present study did not find significant disruption in social interaction for outbred mice in withdrawal; if anything, there was a slight increase from Weeks 0 to 1 for the female stimulus test only, but otherwise no change between weeks for either the Low Drinking or High Drinking groups. The upward trend of the animals to increase in social interaction may have several implications. Though most literature suggests the opposite effect, increased social interaction has been seen in adolescent mice during acute ethanol withdrawal (Varlinskaya and Spear, 2013). Increased social interaction was also seen for mice exposed to chronic social stress and ethanol vapor (Conrad and Winder, 2011). Low ethanol doses have also been found to increase social activity in mice and rats (File, 1980).

At the same time, it is possible that the social interaction test protocol with intermittent access was not successful in screening changes in anxiety-like behavior. First and foremost, the social interaction protocol of this study was adapted both from the original protocol by File in 1983, and by Moy in 2004. This experiment, in order to efficiently assess individual escalating drinking behavior and withdrawal intensity, involved performing the social interaction test several times in the span of 8 weeks, and up to two times within one week. Other studies that use social interaction to screen anxiolytic compounds do so by only employing the social interaction test at one time, not multiple, repeated sessions. Studies that specifically look at withdrawal behavior or as behavioral models for autism or schizophrenia use the social interaction test as a final conclusive test once as a component of a battery of behavioral tests of anxiety like behavior (File, 2003). Most behavioral tests are sensitive to repeated testing, such as the Light/Dark box, or Elevated Plus Maze, which can both be used only once. Though the social interaction has sometimes been used as a repeated measure successfully, there may be an extent to how often the test can be repeated, or how long the inter-test interval must be (Newman, *unpublished*).

One ongoing study that uses the same protocol of repeated social interaction testing has been able to see impaired social interaction over time during withdrawal, but with inbred mice and with dose-dependent drug manipulations (Newman, *unpublished*). In this experiment, C57BI/6J male mice were given several doses of saline vehicle, midazolam or allopregnanolone at different times between 8 to 12 weeks after intermittent access to 20% ethanol. Doses were randomly assigned for each week, but each subject was given each dose by the end of the 12 weeks. The vehicle data (Figure

15) demonstrated that ethanol drinkers spent significantly less time in the interaction zone than control water drinkers. Despite differences in timeline of testing, the disruption does indicate that repeated social interaction can be successful, at least for this inbred strain. It is known that different strains have varying sensitivity to the social interaction test, and it is likely that such sensitivity may be influenced by repeated testing (Olivier and Mos, 1988; Moy, 2004).

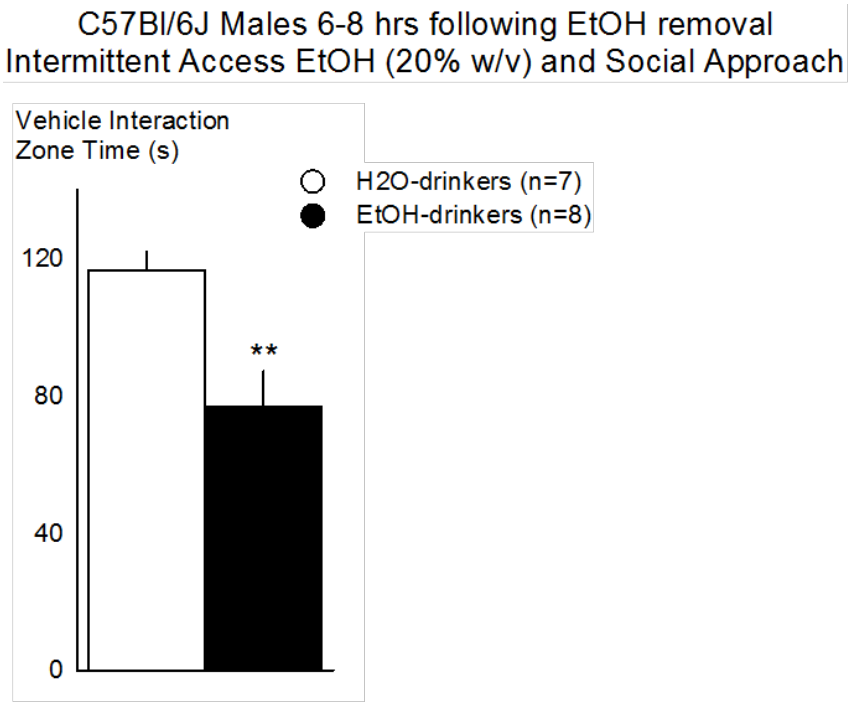


Figure 15: Social Interaction Duration of Inbred Male Mice in Withdrawal
The following figure is taken from a study by Newman (*unpublished*) of C57BI/6J male mice in the vehicle condition for a repeated social interaction test. The testing took place over 8-12 weeks, and found that the Ethanol drinking group (n=8) spent significantly less time in the interaction zone than Water Controls (n=7).

Another factor that may have contributed to the lack of social disruption could be how the subject animals were housed. For the IAA drinking protocol, animals are normally singly-housed in order to assess individual drinking. However, for social

interaction protocols, mice are recommended to be group housed throughout the experiment (Kuti and Page, 2011; van Loo et al, 2003). It is possible that single housing the subject animals may have caused a social deprivation effect that led to more consistent behaviors of social interaction over time. Stress due to single housing has not been inconsequential in studies of anxiety-like behavior and animal behavioral models in general. (Voikar, 2005; Chesler et al, 2002; Crabbe et al, 1999; Valzelli, 1973). Single housing the animals have been shown to modify baseline social interaction behavior and increase variability, which may also explain why even the water control animals experienced high variability week to week (Kuti and Page, 2011; van Loo et al, 2003). Animals that are single housed have decreased anxiety-like behavior in the elevated plus maze, when compared to group housed animals (Voikar, 2005).

One of the unexpected findings of the present experiment was the increase in locomotor activity across all groups over time. Prior studies have found that in the social interaction test, locomotor activity is unchanged between groups, limiting the confounding factor that changes in interaction duration are not due to decreased motor movement at large, and truly due to changes in sociability or anxiety-like behavior (Newman, *unpublished*; File, 2003; Conrad and Winder, 2011). However, though this study was contrary to much of the current evidence on locomotor activity in the social interaction test, there may be several points of consideration to understanding these results. First, the increased motor movement may in fact be linked to glutamate hyperexcitability. As previously mentioned, altered glutamate levels have been associated both with ethanol intake as well as withdrawal; notably, ethanol withdrawal has been linked to glutamate hyperexcitability (Chefer et al, 2011; Follessa and Ticku, 1996; Grant

et al, 1990; Rossetti et al, 1999). This hyperexcitability may be the cause of the increased motor activity (Keele, 2005).

Alterations in locomotor activity are not novel to ethanol withdrawal studies. Prior studies have shown ethanol induced motor impairment and sedative effects in animals, which varies in sensitivity depending on age and strain (Hollstedt et al., 1980; Little et al, 1996; Moy et al, 1998; Silveri and Spear, 1998). Locomotor activity and ethanol-stimulated activity are both correlated to some extent to ethanol withdrawal severity, but is also associated to strain type (Metten and Crabbe, 2005). Additionally, locomotor activity induced by ethanol sensitization has been found to impact only subgroups of animal subjects, suggesting that there are other components to determining this behavior, and that not all animals experience sensitization to the same extent, if at all (Nona et al, 2014).

The main problem with the idea that ethanol withdrawal or intake caused this locomotor effect is that control animals also significantly increased in locomotor activity, though they were not exposed to ethanol. It is also possible that single housing the subject animals may have also contributed to their increased locomotor activity, as well as social interaction. Animals that have been observed to have increased motor movement, as well as increased frequency in movement between chambers or abnormally long durations in one spot in the chamber, may in fact be stressed (Kuti and Page, 2011). Isolated mice have been shown to exhibit hyperactivity in several behavioral measures, including elevated plus maze, light/dark box and others (Abramov et al, 2004; Hlakivi et al, 1989; Rilke et al, 1998; Voikar, 2005).

Limitations and Future Considerations.

Though the present study was not able to confirm the results of previous studies on social interaction and withdrawal, it does raise several important questions for future research. Choice of strain of the subject animal is essential. While it is translationally useful to use an outbred strain, such as in this present study, there is also much variability in behavior and in escalation of dependence for ethanol (Metten and Crabbe, 2005; Crabbe, 2002; Oroszi and Goldman, 2004; Wall et al, 2000). In order to assess dependence, an inbred high drinking strain, such as C57BL/6J, would guarantee high levels of ethanol drinking, and therefore, apparent withdrawal symptoms (Hwa, 2011). Additionally, strain type can dictate baseline sociability, and more importantly, how well subjects can perform in behavioral measures, including social interaction (Metten and Crabbe, 2005; Voikar, 2005). Some strains of mice are less suited than others for single tests of social interaction, and others may be even less so for repeated tests of social interaction. Currently, very little research exists that explores the intersection of repeated social interaction testing and strain differences, and this study may provide one piece of information to that effect. Additionally, the single housing stress is found to impact strains of animals differently and influence how they perform in behavioral tests (Voikar, 2005).

The present protocol may also be adjusted for future studies involving ethanol withdrawal. Due to the increase in locomotor activity across all subjects, including controls, it is likely that the overall protocol led to the overall results, and was not solely due to the withdrawal from ethanol. IAA requires single housing of subjects and voluntary consumption, both of which may have contributed to results of this study.

Utilizing another ethanol administration paradigm, such as the ethanol vapor chamber, would certainly diminish the translational quality of the study, but it might also allow for animals to be group housed during the intake period. The effect of repeated social interaction testing may also be determined by allocating one group of subjects to be test only once at a specific week of withdrawal, instead of repeatedly at each time point as done in this study. Though this is not ideal in terms of resources or time, it would confirm if it was repeated testing sessions that contributed to these results for these outbred mice. Additionally, the time point for the baseline measurement may also be adjusted. In this protocol, baseline was recorded a week after habituation to laboratory conditions while subjects were single housed and in one test session; baseline values may be less variable and more comparable if perhaps multiple baseline tests were performed before alcohol administration and averaged to diminish some of the novelty of initial single housing stress.

Another recommendation is enhancing how social interaction is measured. For this study and others, Noldus tracking software is used to track movements for each testing session and quantify results by duration, distance, frequency, velocity and other measurements based on what the researcher is studying. However, while these measures are valid, they may also overlook particular social behaviors by lumping all movements together. Prior studies have scored social interaction based on a variety of behaviors, including sniffing, play fighting, and grooming, each of which can differ in frequency for subjects across different ages, sexes, or strains (Nadley et al, 2004; Varlinskaya and Spear, 2013). It is possible that by not scoring individual behaviors, information about changes in these behaviors is overlooked. Some studies have shown that other behaviors

may be more or less sensitive to change compared to social interaction, such as freezing, self-grooming, defecation, rearing, exploration, and locomotor activity, which is why they may also be important to study individually (File and Hyde, 1978, File and Seth, 2003). Additionally, as many of these behaviors are thought to be mediated by different systems, it is possible that the social interaction test may reveal one or none of these behaviors, but that other behavioral measures might be more sensitive to others (Varlinskaya and Spear, 2013).

Ultimately, use of social interaction to understand anxiety like behavior during withdrawal from ethanol has a place in understanding withdrawal severity. The results of this study provide context for considering numerous other factors of both the test and the administration of ethanol that have largely been overlooked. Ongoing social interaction studies should critically examine the effect of these factors in order to more conclusively understand how these behaviors are contingent on conditional circumstances.

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Appendix

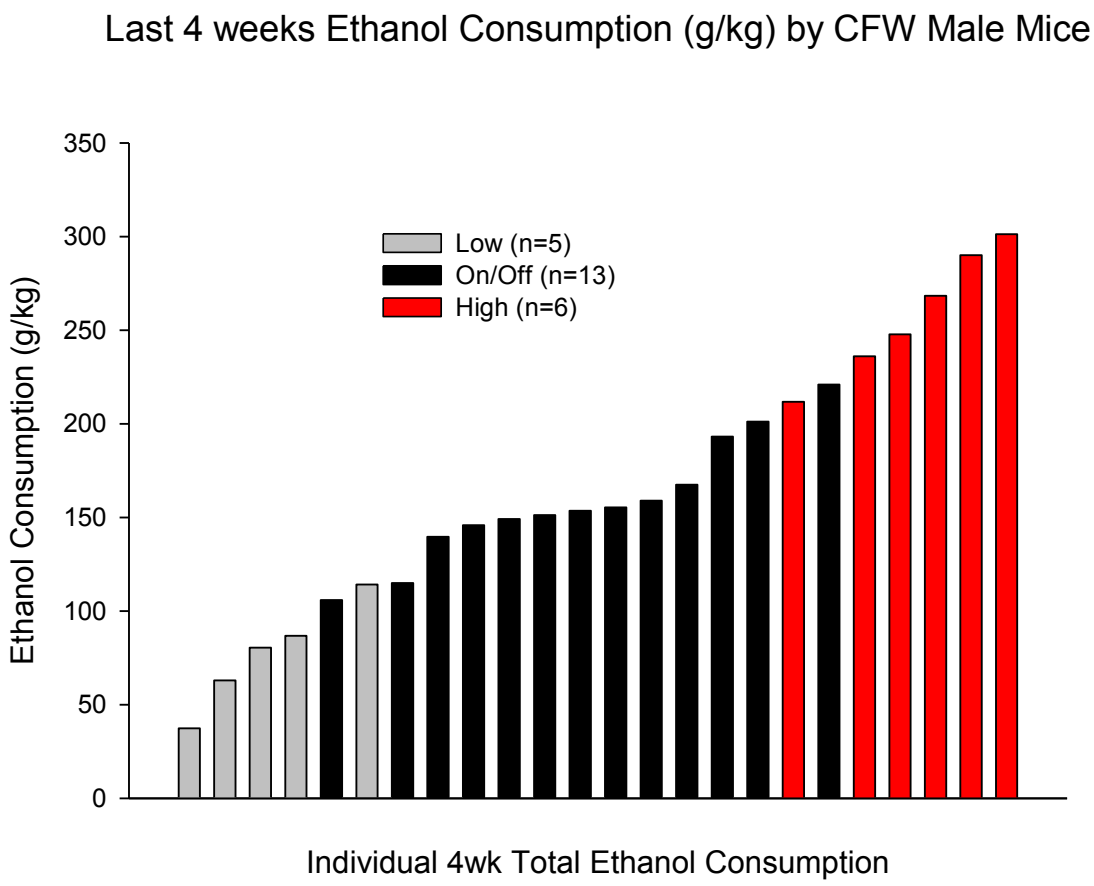

Figure 3: Total Ethanol Intake of Last 4 weeks of IAA by Outbred Male Mice
In this figure, the total intake of ethanol in g/kg for the last four weeks of intermittent access to 20% ethanol by CFW Male mice is seen. After total 4 week intake was arranged from least to greatest intake, a criteria was established to separate the groups into Low Drinking (n=5), High Drinking (n=6) and On/Off Drinking groups (n=13), which are described in the legend above.

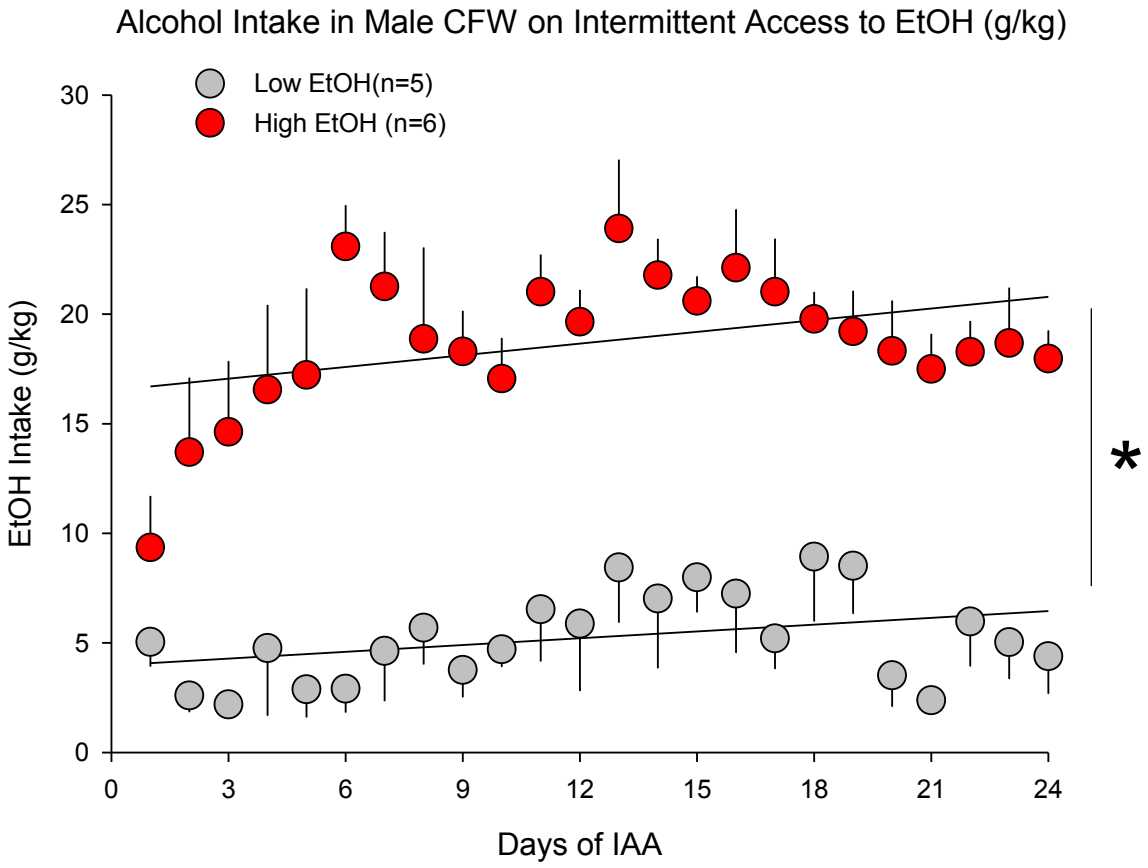

Figure 4: Daily IAA Alcohol Intake by CFW Male Mice

In the above figure, groups of CFW male mice were separated into a High drinking and Low Drinking group (see *Methods-Statistical Analysis* for criteria). Intake was recorded in g/kg for every intermittent access to 20% ethanol day. The Low Drinking group (n=5) and High Drinking group (n=6) were statistically different in intake ($p<0.001$), but not over time. The On/Off group are omitted here.

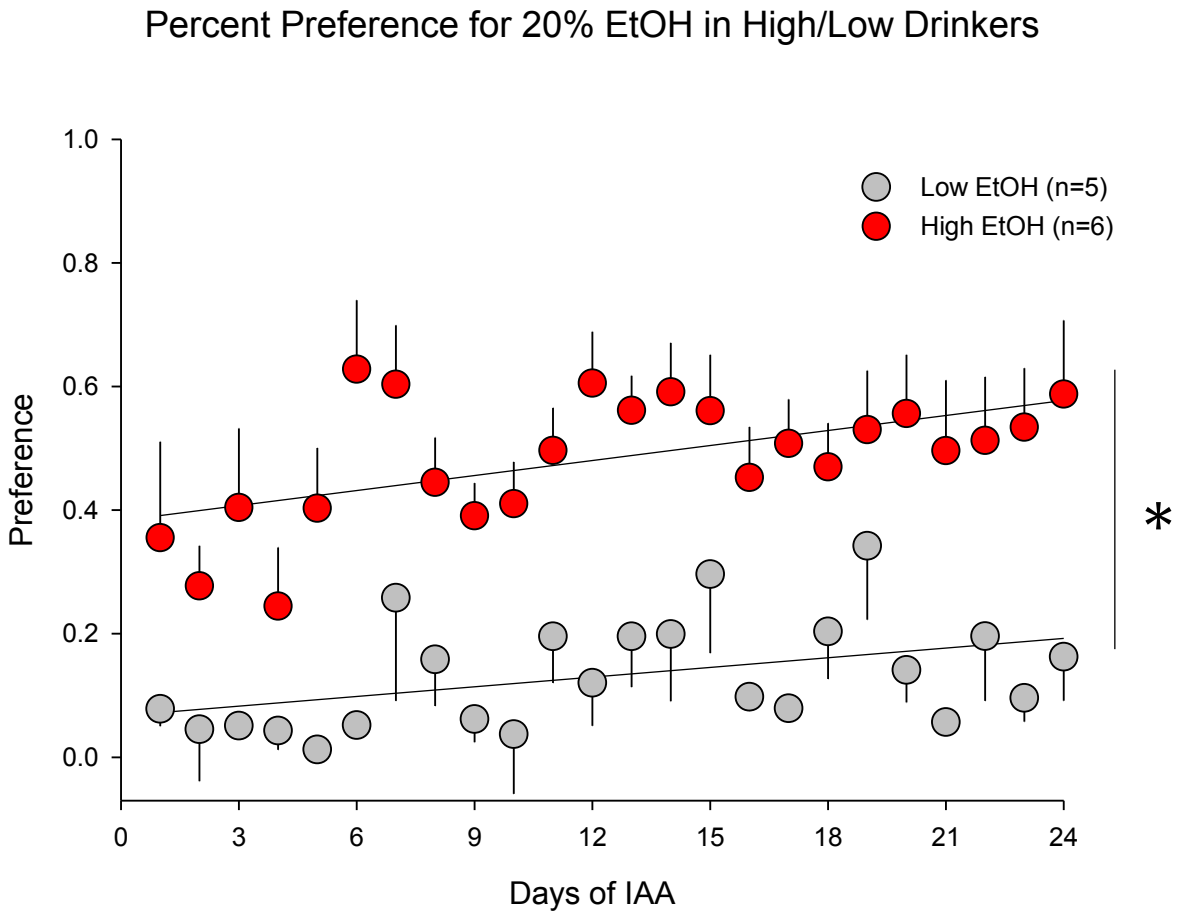

Figure 5: Percent Preference

The figure here again separates the High Drinking (n=6) and Low drinking (n=5) groups by the same criteria as seen in Figure X. Percent preference was calculated with amount of 20% ethanol consumed over total fluid intake. The two groups differed significantly in preference for ethanol ($p<0.001$), but again no effect was seen over time.

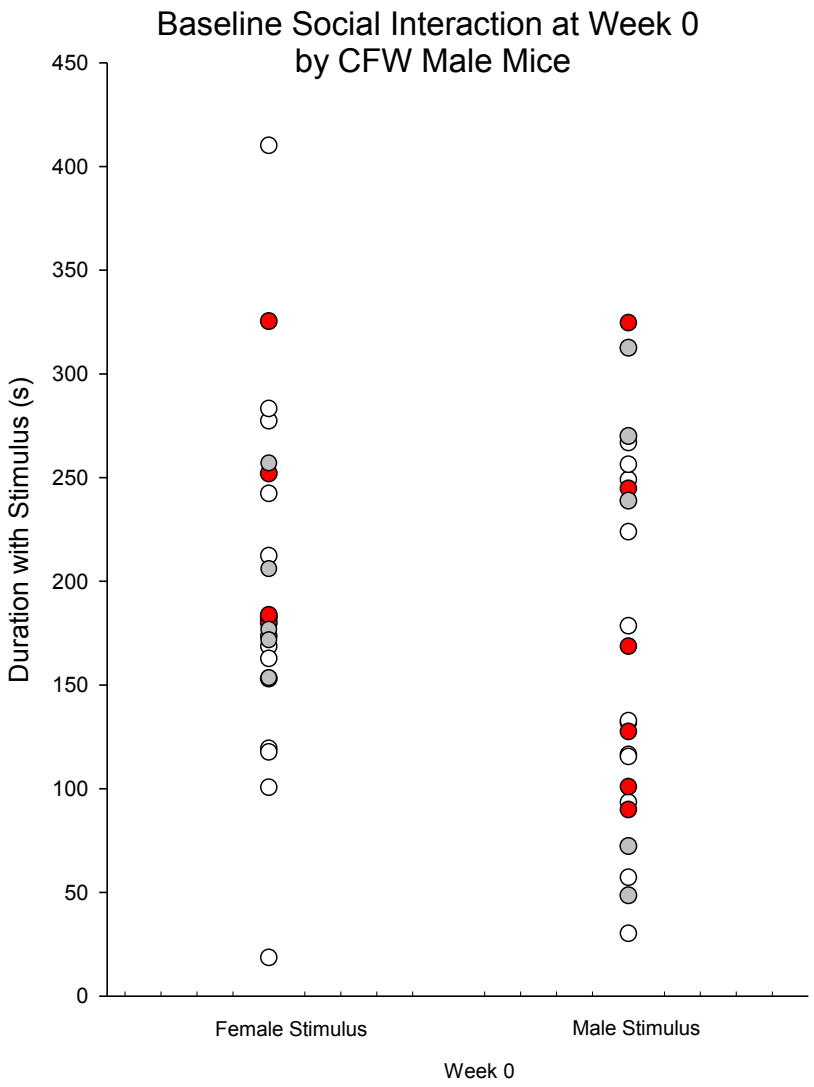

Figure 6: Individual Baseline Measures for Social Interaction Test

The above figure shows the individual baseline measures taken at Week 0 before IAA began. Measures were taken as duration of time in seconds spent with either the female stimulus or male stimulus animal in the interaction zone.

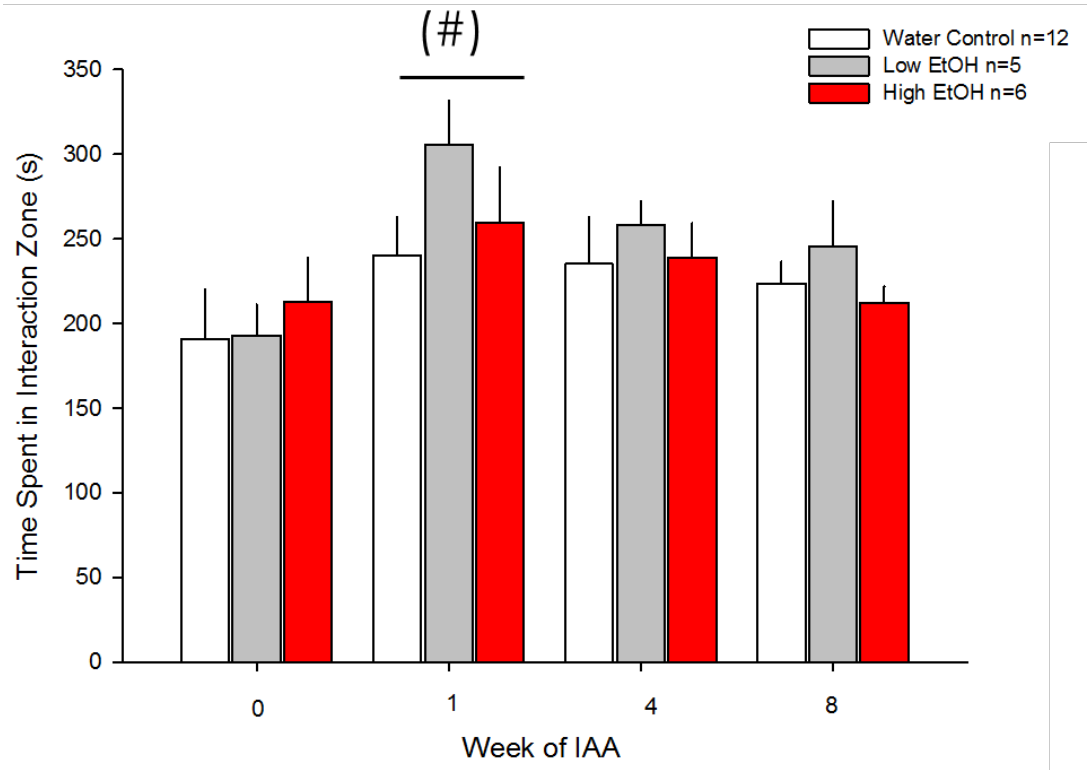

Figure 7: Social Interaction of Male CFW Mice with Female Stimulus

In this figure, the social interaction test was measured by duration of time spent in the interaction zone in seconds with the female stimulus at several time points in withdrawal. Groups were separated into High drinking (n=6), Low drinking (n=5) and Control group (n=12). Overall no significant effect was found, but there was a trend-like pattern seen between weeks 0 and 1 for all groups, as denoted by the # sign (p=0.071).

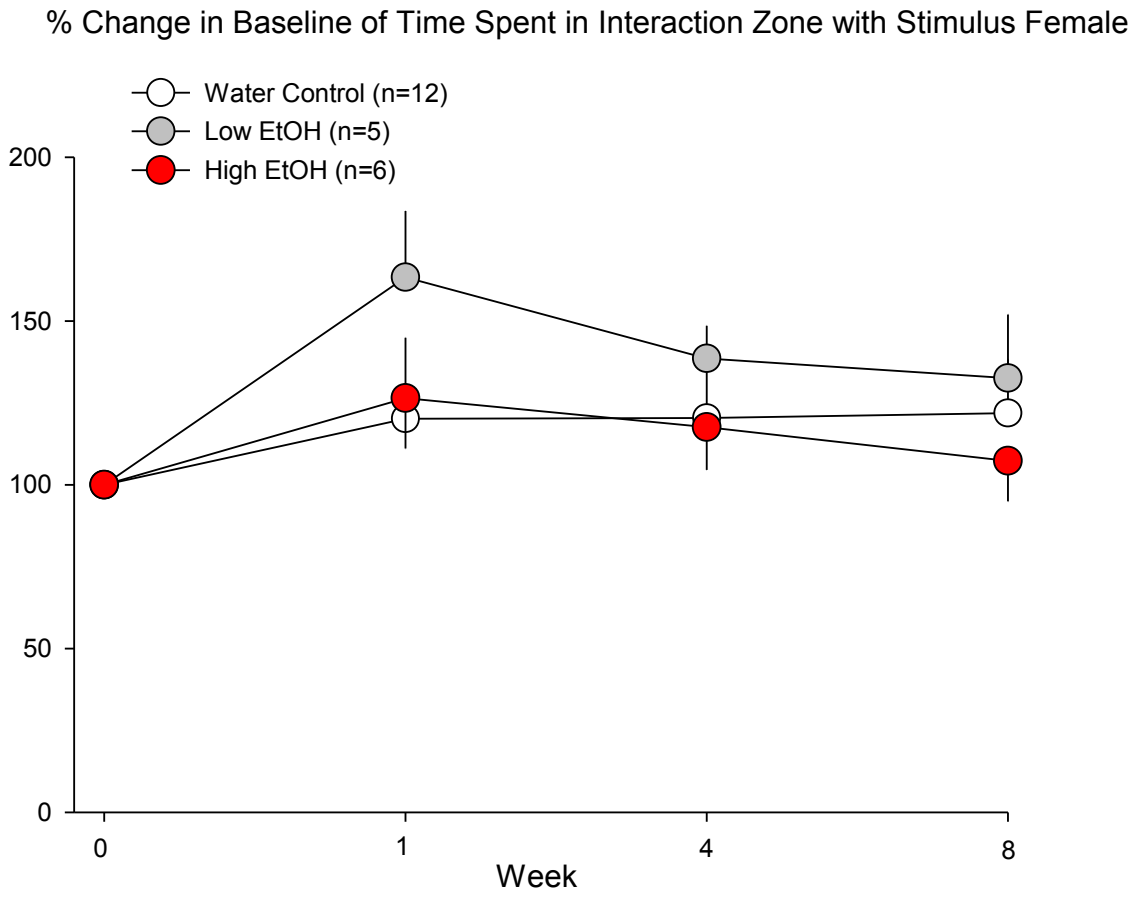

Figure 8: Percent Change from baseline for Social Interaction of Male CFW Mice with Female Stimulus

The above figure demonstrates the percent change from baseline in social interaction with the female stimulus animal across weeks 1, 4, and 8. No significant effect was found over time or between groups.

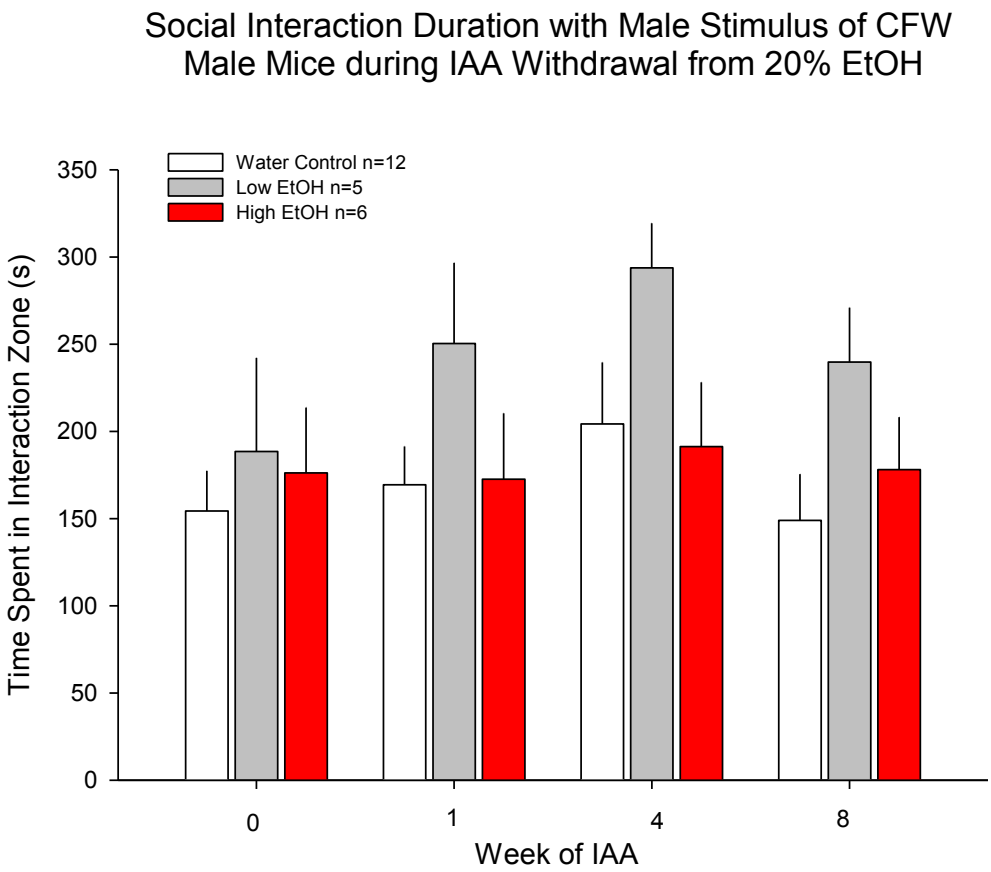

Figure 9: Social Interaction of Male CFW Mice with Male Stimulus

In this figure, the social interaction test was measured by duration of time spent in the interaction zone in seconds with the Male stimulus at several time points in withdrawal. Groups were separated into High drinking (n=6), Low drinking (n=5) and Control group (n=12). Overall no significant effect was found.

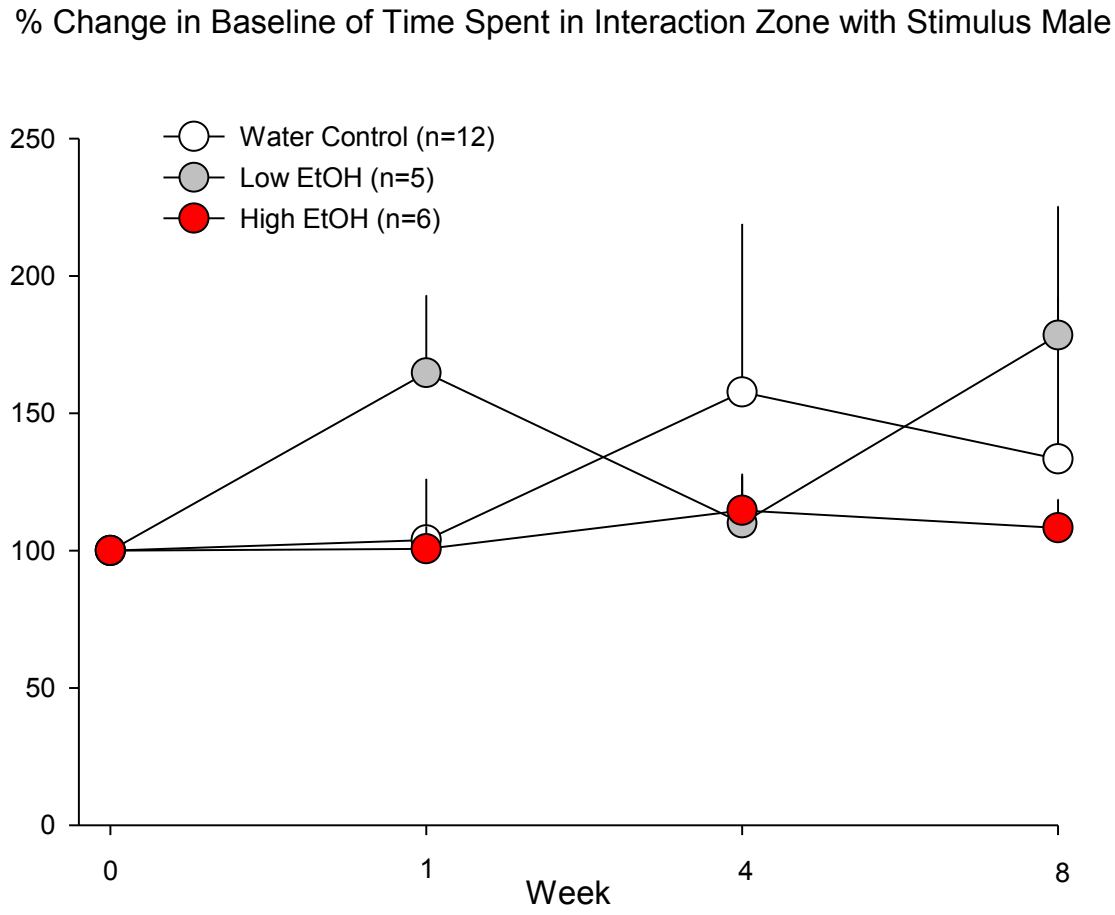

Figure 10: Percent Change from baseline for Social Interaction of Male CFW Mice with Male Stimulus

The above figure demonstrates the percent change from baseline in social interaction with the female stimulus animal across weeks 1, 4, and 8. No significant effect was found over time or between groups.

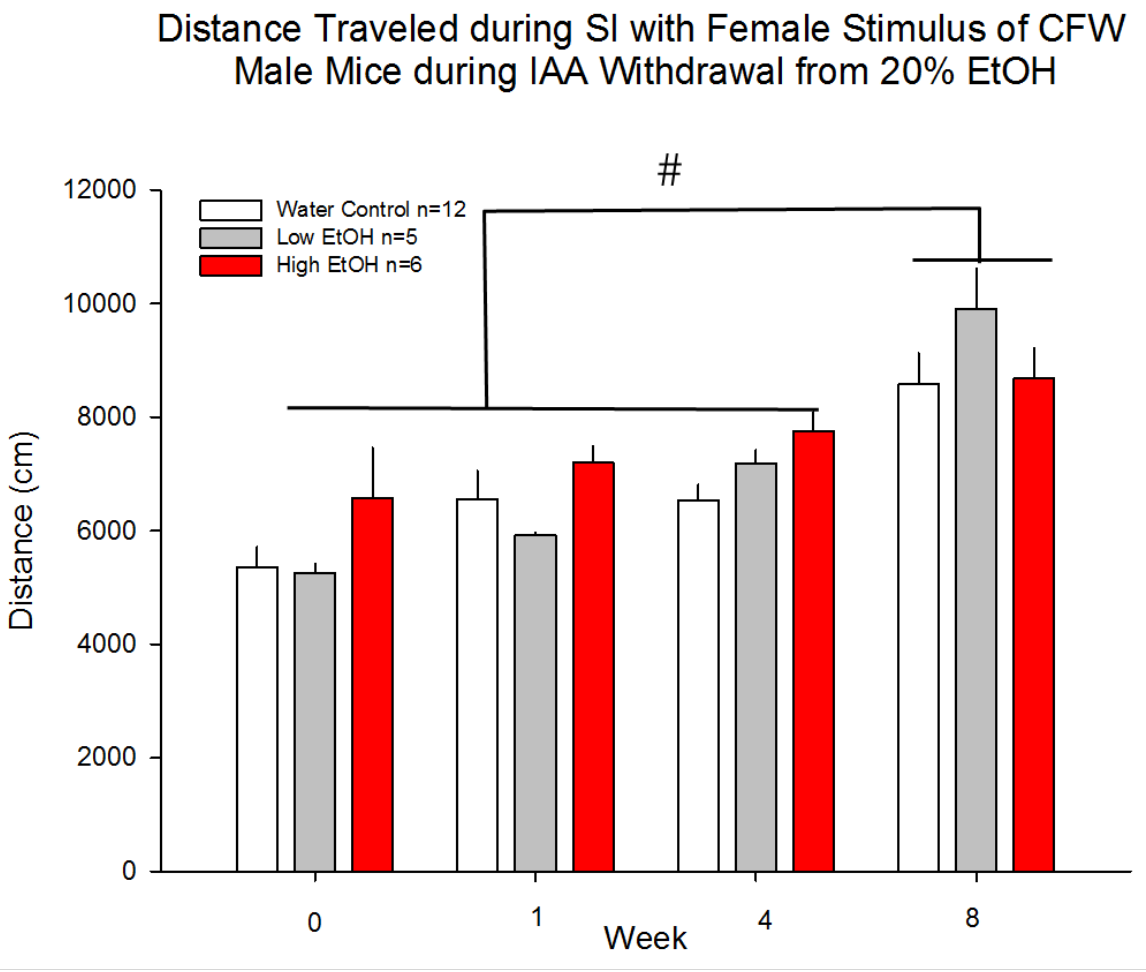

Figure 11: Locomotor Activity of CFW Male Mice during Ethanol Withdrawal
The figure describes the change in locomotor activity seen in outbred CFW male mice during social interaction test with a female stimulus. Locomotor activity was significantly increased in Week 8 compared to baseline (Week 0), Week 1 and Week 4, across all three groups (p<0.001).

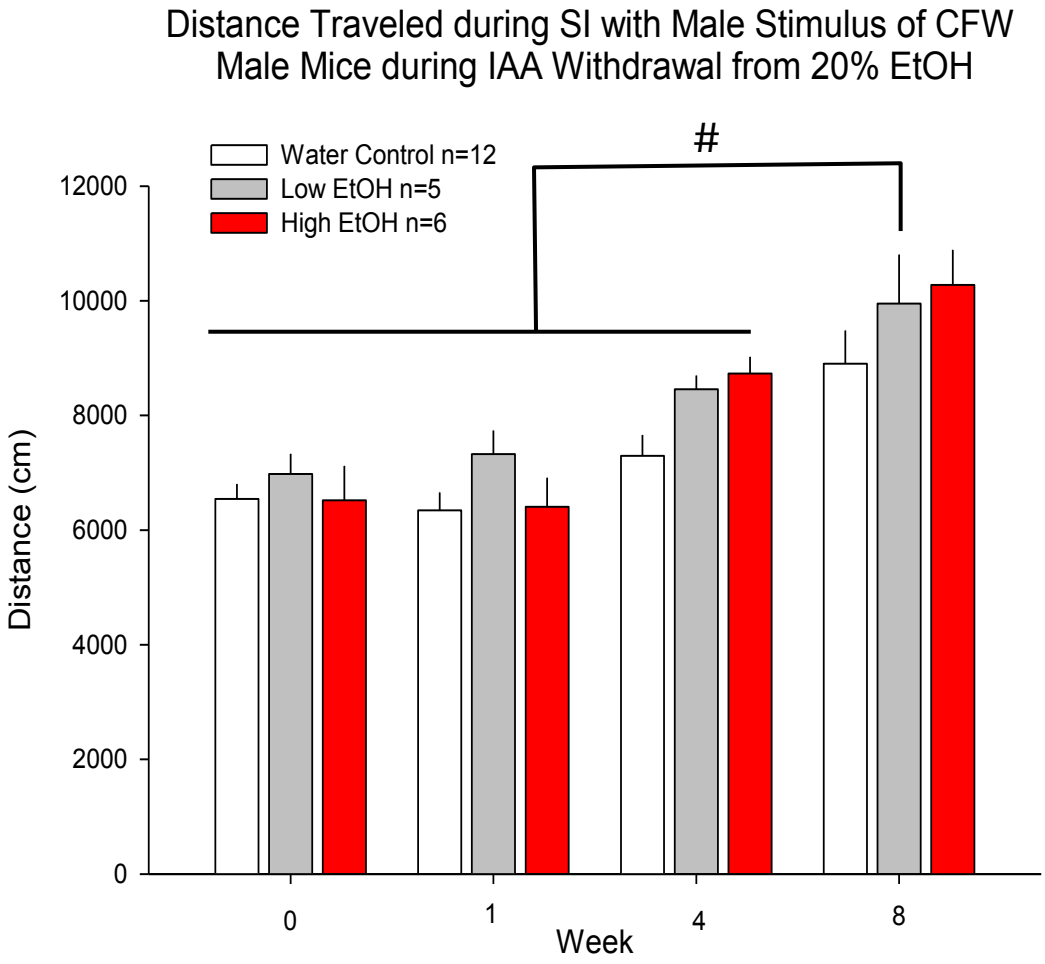

Figure 12: Locomotor Activity of CFW Male Mice during Ethanol Withdrawal

The figure describes the change in locomotor activity seen in outbred CFW male mice during social interaction test with a male stimulus. Locomotor activity was significantly increased in Week 8 compared to baseline (Week 0), Week 1 and Week 4, across all three groups ($p<0.001$).

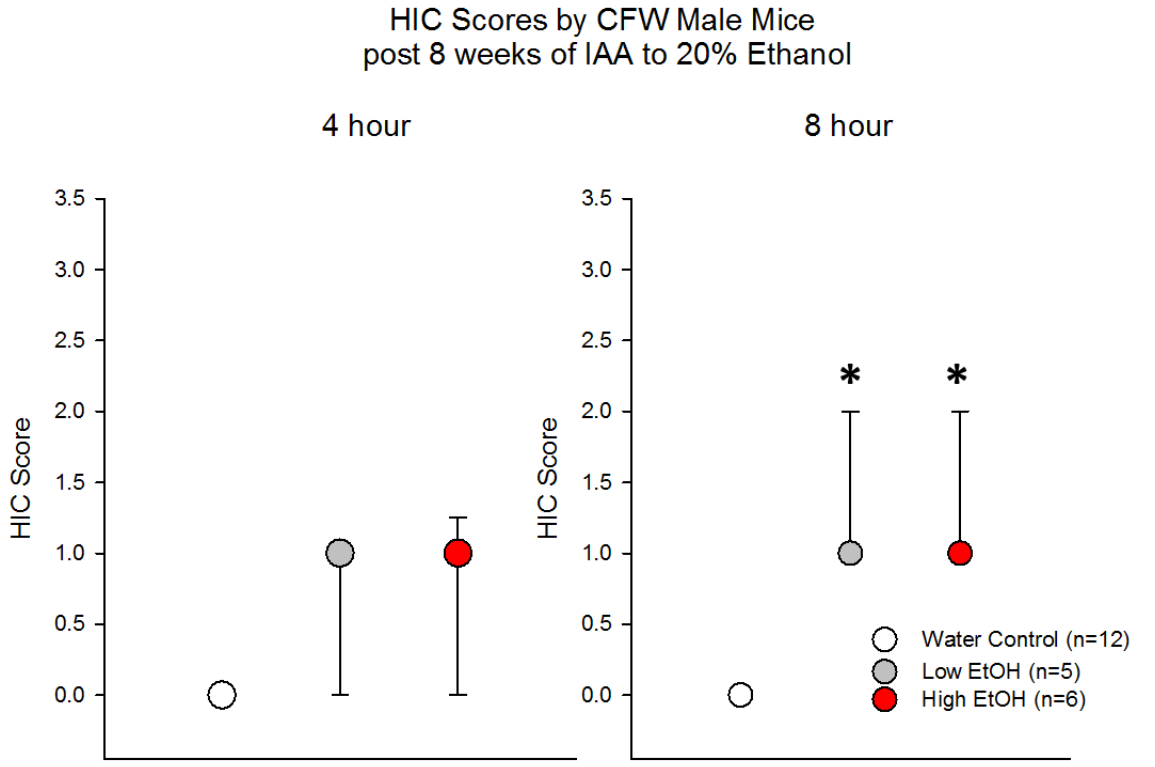

Figure 13: HIC Scores for CFW Male Mice

The following figure describes HIC scores for CFW male mice post 8 weeks of intermittent access to 20% ethanol for the High (n=6) and Low (n=5) Drinking groups and Control (n=12) group. HIC scores were taken 4 hour and 8 hours in withdrawal. The asterisk notes significant difference from the control group for both the High and Low Drinking groups (p<0.001).

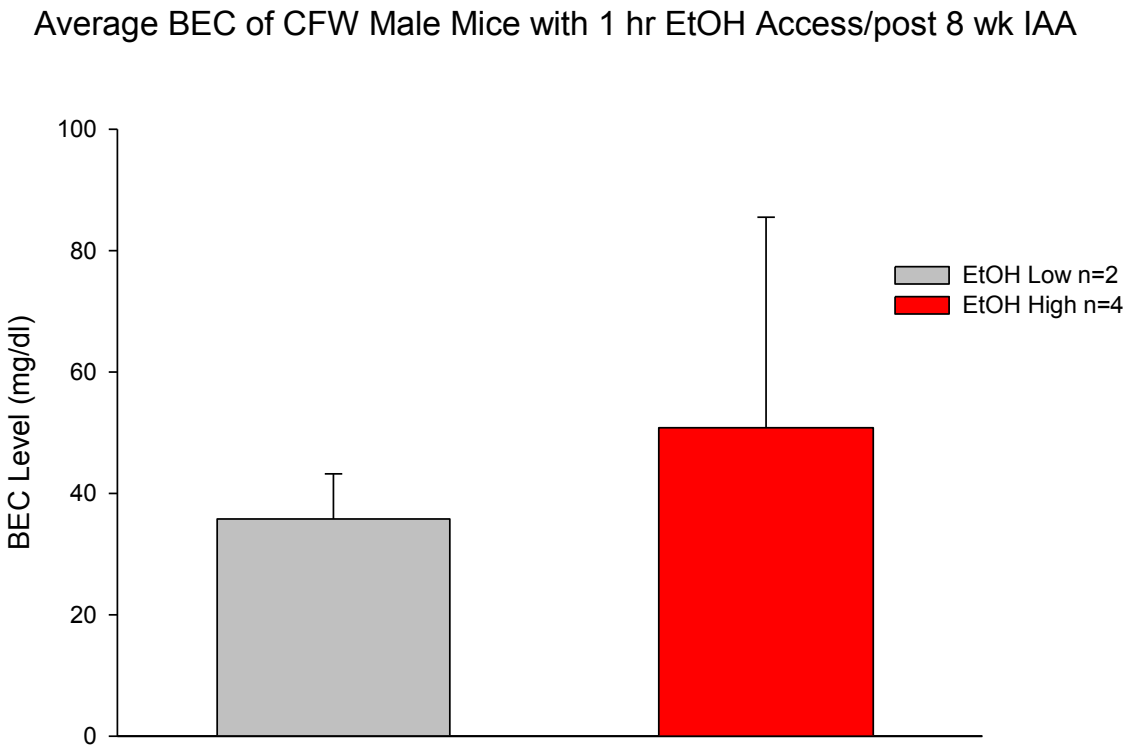

Figure 14: Average BEC level for CFW Male Mice

The figure above describes the average BEC level for each the High Drinking (n=2) and Low Drinking (n=4) groups. BEC levels were determined on samples collected 1 hour after access to 20% ethanol post 8 weeks of IAA. No significant difference was found between the groups.