Role of CRF-R1 in social defeat stress escalated voluntary ethanol consumption in CFW mice

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Abstract

Stress can play a role in alcohol and substance abuse, but the neurobiological mechanisms that underlie this relationship are unclear. The current study pharmacologically tested the role of corticotropin-releasing factor type-1 receptors (CRF-R1) in ethanol drinking following social defeat stress. Adult, male CFW mice were exposed to either 15 or 30 attack bites for 10 consecutive days using the resident/intruder paradigm. Ten days after the final social defeat, mice were tested for the expression of locomotor sensitization following a 2 g/kg intraperitoneal (i.p.) ethanol challenge. Daily, voluntary 20% ethanol and water intake was then measured during a three week period of continuous access to two bottle free choice. Doses of a CRF-R1 antagonist, CP-376,395 (10, 17, and 30 mg/kg, i.p.), were administered, and ethanol and water drinking were recorded 2, 4, and 24 hours after drug administration for stressed and non-stressed mice. We found that mice that experienced the severe (30 attack bites) stress, but not the moderate (15 attack bites), during social defeat stress period consumed more ethanol than non-stressed controls. This severe stress protocol had no effect on previously acquired ethanol drinking. CP-376,395 decreased ethanol intake of the high ethanol drinkers in a dose-dependent manner during the initial 2 hours compared to the low ethanol drinkers; after 4 hours the drug effect was only observed at the highest dose. This pattern suggests that more intense and longer social stressors escalate ethanol consumption, but not lower intensity and shorter duration stressors. Previous exposure to ethanol access results in a delayed stress-induced escalation of ethanol drinking. CRF-R1 may play a critical role in mediating stress-induced ethanol consumption. Ongoing studies using an operant conditioning procedure suggest that socially defeated CFW mice have increased motivation for alcohol. It is anticipated that lower HPA axis

activation and limbic CRF and dopamine will differentiate the stress mechanisms that engender escalated alcohol consumption.

Stress and Alcoholism

Approximately one-third of people have turned to alcohol to relieve stress, although the motivation for individuals to drink alcohol varies (BBC News, 2012). Stress is a stimulus that disrupts the homeostasis of the body and forces physiological adjustments to adapt to the stressor, usually at the expense of an individual's health (Selye, 1936). Whether it's a national disaster, the loss of a loved one, or the chronic worrying about financial woes, stress is a part of everyone's life. Individuals that experience anxiety, a major cognitive symptom of everyday stressors, often choose alcohol to cope with their problems and anxiety, according to the tension reduction hypothesis (Conger, 1956). The interaction between stress, anxiety, and alcohol abuse is complex and more research is required to further understand this relationship.

The use of alcohol as a coping mechanism for anxiety often leads to health problems and alcohol use disorders (Cappell & Greeley, 1987). According to the Diagnostic and Statistical Manual, 5th Edition (DSM-5, 2013), an alcohol abuse and dependence diagnosis requires at least 2 out of 11 specific symptoms including, alcohol use resulting in the inability to perform everyday activities at work and drinking excessively for prolonged periods. In 2012, U.S. men and women that reported between 0 and 6 major stressors in the past year showed increasing rates of alcohol drinking, binge drinking, and alcohol use disorders (Keyes et al, 2012). Approximately 50% of men and 33% of women that experienced 9 major stress events in 2012 were diagnosed with an alcohol use disorder.

A strong relationship between clinical populations of high anxiety, such as those with agoraphobia and social phobia, and alcohol abuse as a coping mechanism exists (Quitkin et al, 1972). In these cases, there is high prevalence of alcohol abuse among those with a lifetime

anxiety disorder, usually as an attempt to decrease the stress caused fear of new places or social interactions (Schuckit & Hesselbrock, 1994). In addition, alcohol has been shown to decrease measures of behavioral and autonomic anxiety during a social interaction (Abrams et al, 1980). Chronic stress has been determined to be a major trigger point to the development of anxiety disorder.

Stress-related disorders like post-traumatic stress disorder (PTSD) are major risk factors for alcohol abuse. PTSD is a mental disorder that develops after traumatic events and is characterized by flashbacks of the event and hypervigilance that persist for months after the trauma (DSM-5, 2013). As with a large proportion of individuals that experience everyday stressors, individuals with PTSD often cope with the stress by self-medicating with alcohol. A National Comorbidity Survey showed that 52% of men and 28% of women with PTSD had also been diagnosed with alcohol use disorder (Kessler et al, 1995). PTSD and alcoholism have a high prevalence in combat veterans, as over 20% of soldiers returning from recent wars in Iraq and Afghanistan have met the requirements for PTSD and 27% of returning veterans are diagnosed with an alcohol use disorder (Spoont et al, 2013; NIDA, 2011). While epidemiology studies show a clear relationship between this psychiatric disorder and alcohol abuse, less evidence exists for a biological explanation of this relationship.

Animal models of alcohol drinking and stress can be used to further investigate the biological mechanisms. Validated models of stress that activate the hypothalamic-pituitary-adrenal (HPA) axis are characterized by neuroendocrine stress response, shown by elevated levels of glucocorticoids. While it was been shown that stress plays a role in alcohol addiction, the biological mechanisms that underlie this relationship are less understood.

Animal models of Ethanol Drinking

The distinct physiological and behavioral changes involved in alcoholism can be assessed by using a variety of animal models of drinking (McBride & Li, 1998). Although no animal model can perfectly translate to alcoholism in humans, partial models pertaining to specific aspects of alcoholism can be developed (Rhodes et al, 2005). The manner in which the rodent is exposed to ethanol is a key determinant of the conclusions that can be made from the study. An animal model that produces reliable and consistent significant ethanol drinking, with blood ethanol concentration (BEC)>1.0mg/ml, must be established in order to assess the efficacy of experimental pharmaceutical compounds in decreasing ethanol drinking (Rhodes et al, 2005). These translational models of drinking can be used to further explore the genetic and neurological basis of alcohol addiction in the clinical population (Kamdar et al, 2007).

A variety of animal models of ethanol drinking are described in the following table. Not all models guarantee alcohol consumption that produces alcohol dependence level BECs, but each model has individual benefits and limitations to the design. The optimal experimental design may vary depending on whether acute effects of ethanol, motivation for ethanol reward, behavior during withdrawal, or pharmacological manipulation following long-term access to ethanol are critical aspects of the study. Multiple models of alcohol drinking can be used to strengthen the predictive validity of the efficacy of an experimental compound. In the current study, mice were given continuous access to ethanol and water, despite evidence that this procedure has been shown to produce lower BECs and not induce handle-induced convulsions during withdrawal, a staple sign of alcohol dependence (Hwa et al, 2011). A major benefit of using this procedure to study stress and alcohol drinking is that it avoids the withdrawal period that exists in many

models. The stress of withdrawal has been shown to increase ethanol intake and this stress could potentially interfere with interpretations of social stress and alcohol drinking that is explore in this paper (Heyser et al, 2003).

Table 1. Assessment of Animal Models of Ethanol Consumption

Method	Description	Main Advantages	Main Disadvantages	Blood Ethanol Concentration (BEC)	Citation
Ethanol diet	Only access to ethanol	Creates elevated ethanol drinking	Does not assess voluntary intake	100-150mg/dl	Lieber et al, 1963
Vapor chamber	Episodic, forced ethanol vapor inhalation	Easy to establish dependence and withdrawal Maintains consistent blood alcohol levels	Involuntary Most human ethanol consumption is not via inhalation	200-300mg/dl	Goldstein & Pa, 1971; Griffin et al, 2009
Two bottle choice (intermittent access)	Episodic presentation of alcohol	Mimics repeated cyclical alcohol access and deprivation	Difficult to demonstrate motivation	79-167mg/dl	Wise, 1973; Hwa et al, 2011
Sucrose fading	Sweetener (saccharin) is added to alcohol Over time, sweetener concentration is decreased and alcohol concentration is increased	Increase ethanol consumption of rodents that otherwise wouldn't	Cannot assess taste aversion Alcohol is not naturally sweet	40-110mg/dl	Samson, 1986
Drinking in the dark	Limited access to ethanol during high drinking period	Rodents show ethanol "binge" behavior Intoxication	Limited access to alcohol	0-250mg/dl	Rhodes et al, 2005

Outbred Cartworth Farm Webster (CFW) mice were chosen for this experiment, because of a combination of their social nature and preference for alcohol. CFW mice have been shown to drink pharmacologically relevant levels of alcohol and will also self-administer for alcohol in a nose-poke task (Faccidomo et al, 2008; Takahashi et al, 2010). It is important to note that CFW mice drink in multiple animal models of alcohol drinking including intermittent or continuous access to ethanol and water and ethanol as well as self-administration (unpublished). This adds versatility to the different experiments that can be performed. The strain of mice is outbred, so there is an increased chance of individual differences in physiology and behavioral that can be further explored. CFW were also chosen to be 'resident' mice as they have been shown to be reliably aggressive at attacking intruder mice that have been introduced into their cage (Miczek & O'Donnell, 1978).

Sensitization to Alcohol

Sensitization occurs when repeated exposure to equivalent stimuli cause an increase in response, and this is believed to play a major role in addiction (Robinson & Berridge, 1993). Sensitization can be operationally defined multiple ways, such as neuronally by increased neurotransmitter release or behaviorally by increased locomotor activity. The 'Incentive-Sensitization theory,' proposed by Robinson and Berridge, suggests that addiction can develop from associative learning between drugs and related stimuli that potentiates drug craving behavior. The sensitization phenomenon was first discovered in 1932 with the use of repeated injections of amphetamine, but cocaine and morphine have been shown to be capable of similar effects (Downs et al, 1932; Post et al, 1991). There is evidence of a genetic component of sensitization to alcohol in clinical populations, as finger pulse levels of sons of alcoholics

showed sensitization to repeated injection of alcohol (0.5g/kg) while sons of non-alcoholics did not show this effect (Newlin & Thomson, 1991). In mice, daily injections of ethanol (i.p.) were given for 5 consecutive days and increased locomotor activity was seen following an ethanol injection after 10 days drug-free days (Itzhak and Martin, 1999). A selected line of high-alcohol preferring mice, but not low-alcohol preferring, showed locomotor sensitization to ethanol challenges (2.75, 3.5g/kg) following four repeated injection at 48 hour intervals, suggesting that there is a strong relationship between the ethanol sensitization and consumption (Grahame et al, 2000).

Cross-sensitization occurs when repeated exposure to one type of addictive drug causes an individual to be become sensitized to a different drug (Akimoto et al. 1990; Cunningham & Kelley, 1992; Dafny & Yang, 2006). Cross-sensitization can also occur between repeated exposure to a negative stimulus, such as stress, and drugs of addiction (Robinson & Berridge, 1993). Outbred CFW mice given repeated injections of ethanol showed decreased locomotor activity following a morphine challenge (5mg/kg), showing sensitization to the sedative effects of morphine (Fish et al, 2002). Cross-sensitization can also occur between repeated exposure to a negative stimulus, such as stress, and drugs of addiction (Robinson & Berridge, 1993). Stressors, such as foot shock and food deprivation, have been shown to induce sensitization in response to amphetamine injection (Antelman, 1988). Other studies have shown that a single social defeat experience can invoke sensitization to cocaine and repeated exposure to stress magnifies this effect (Miczek et al, 1999; Covington & Miczek, 2001).

It is important to note that individual differences play a significant role in sensitization to drugs of addiction. Comparison between cocaine sensitization of C57Bl/6J, A/J, BALB parental mice and their mixed recombinant strains revealed that genetics are play a key role in this

behavior (Shuster et al, 1977). Genetics, sex, hormones, and environment are all considered to be significant contributors to individual differences in behavioral responses to drugs of addiction. The use of outbred CFW mice allowed for investigation of individual differences in biochemical and behavioral response to social stress.

Animal models of stress

Physical and psychological stressors trigger the activation of different pathways in the brain and cause differences in ethanol drinking behavior. The interaction between stress and alcohol drinking has been studied using multiple types of physical stress models, including electrical foot shock, immobilization, and forced swimming stress. Previous literature showing the effect of physical stressors on alcohol intake is inconsistent (Becker et al, 2011). When rats were placed into chamber with an electrically charged wire floor and received randomized electric shocks, it triggered an increased voluntary ethanol consumption of 5% ethanol compared to unshocked rats (Volpicelli et al, 1990). In this study, rats that had been selectively bred for low alcohol preference increased intake after a series of unavoidable shock procedures, while genetically-selected high alcohol preferring rats decreased their alcohol intake in response to this physical stressor. These studies suggest that there were bidirectional differences between the alcohol intake of high and low ethanol preferring rats during access to alcohol. Immobilization and restraint stress are potent stressors that rats fail to habituate to after repeated exposure (Kasuga et al, 1999). The effects of immobilization stress on ethanol drinking in rats are also unclear, as some studies show that a 15 minute period of restraint increases ethanol preference, while many others shows a decrease or no change in ethanol consumption (Lynch et al, 1999;

Spraque & Maickel, 1994). It is important to note that restraint stress has not been shown to result in any change in ethanol intake in mice (Chester et al, 2006; Tambour et al, 2008).

Table 2. Criteria for evaluating animal models. (Koob, 2000)

Reliability	The consistency and stability with which the variable of interest is observed
Validity	The criteria for evaluating the usefulness of models
Predictive validity	Ability of behavior in an animal model to predict specific symptom of a human condition
Construct validity	Accuracy with which behavior in an animal model measures what it is intended to measure
Etiological validity	Similarities of the etiology for the behavior exhibited in the animal model and the specific symptoms of the human condition
Face validity	Phenomenological similarity between the behavior exhibited in the animal model and the specific symptoms of the human condition

The differential behavioral and biological response to physical and psychological stressors give reason to why psychological animal stressor models may translate better to the stress that humans experience (Blanchard et al, 2001). Animal models with the high translational value to clinical disease demonstrate multiple aspects of reliability and validity outlined in Table 2. Some models of psychological stress used in animal research are isolation housing, social defeat, and maternal separation. Social isolation can be a sex-dependent model of stress, so the natural social behavior of the animal must be known. Male rats are aggressive and territorial, so being housed with multiple male rats can be more stressful than isolation (Chung et al, 1999). In contrast, female rodents show less aggression when group-housed, but isolation can trigger signs of anxiety-like behavior (Haller & Halasz, 1999). Social defeat procedures involve a confrontation between an aggressive stimulus male and a stressed experimental male. Often, variations of the procedure include a period where the experimental animal is placed in a protective area, in order to maintain stress while reducing the chance of physical injury. When studying the stress response of post-natal rats or mice, a maternal separation procedure can be

used where pups are removed from their mother for a various amount of time (Plotsky & Meaney, 1993). The disruption of this interaction has been shown to alter behavior of offspring and be a stressful experience, as evident by increases in CRF (Viau et al, 1993).

Table 3. Social defeat stress during ethanol access

Stressor	**Species**	**Effect**		**Citation**
		During stress period	Following stress period	
Daily 5min exposed to resident and 30min in protective cage for 5 days	Male Long-Evans rats	Decreased intake of 10% ethanol and self-administration of 15% ethanol during stress	Progressive increase to baseline after stress	Van Erp & Miczek, 2001
5min exposed to resident followed by 30min, 6hr,or 24hr in protective cage (three periods of 5 consecutive days of stress exposure	Male Long-Evans rats	Decreased intake of 10% ethanol during 6hr and 24hr stress, but not 30min	Not available	Van Erp et al, 2001
3 consecutive days- 1 attack by resident followed by 15min in protective cage	Swiss Webster mice	No change in voluntary 8% intake during	Increased ethanol drinking following defeat period	Sillaber et al, 2002
Daily 5min exposed to resident and 30min in protective cage for 5 days	Male Wistar rats	Decreased 12% ethanol self-administration and lever pressing during extinction	Progressive increase to baseline after stress	Funk et al, 2005
5 consecutive daily exposures to resident- 5min or until defeat posture	C57BL/10 mice	Not available	Progressive onset of increased 8% ethanol intake after single defeat	Croft et al, 2005
Social defeat once a week for 4 weeks- 5min or until defeat posture	C57BL/10 mice	No change in 8% ethanol intake	Not available	Croft et al, 2005

Previous studies exploring the effects of social stress during ethanol access in rats and mice have yielded inconsistent results (Table 2). Despite the inconsistency, one common trend is that there is either an increase or no change in ethanol intake during a stress period and ethanol consumption typically increases following the stress period. The results of these studies suggest that if a decrease in ethanol drinking occurred during stress, then ethanol intake will rise to baseline measurements when the stress period is completed (Van Erp & Miczek, 2001; Funk et al, 2005). In contrast, if there is no change in ethanol intake during the stress period, then ethanol consumption has been shown to elevate beyond baseline levels (Sillaber et al, 2002). It is also important to note that consecutive, daily social defeat may be required to induce neuroplasticity changes in the mouse brain that are required for changes in ethanol intake (Croft et al, 2005). This is consistent with research showing that a 5-day chronic social stress model induced long-lasting *c-fos*, a biomarker for cellular activity, in the hypothalamus, amygdala, and hippocampus, while a single, acute exposure to social stress didn't cause such changes (Matsuda et al, 1996). It is clear that a more detailed analysis of the individual social defeat exposures must be assessed to interpret individual differences in the response to stress.

Hypothalamic-pituitary-adrenal (HPA) Axis

In response to a stressor, the HPA axis is activated and the brain sends efferent signals via the sympathetic nervous system (Gold et al, 1998). Activation of the paraventricular nucleus of the hypothalamus results in release of CRF, leading to the stimulation of the hormonally controlled anterior pituitary. The anterior pituitary then secretes adrenaocorticotropic hormone (ACTH) into the bloodstream, where it eventually causes the release of glucocorticoids, corticosterone (CORT) in rodents, from the adrenal glands. Increased glucocorticoids serve as

the basis for the physiological changes during the "fight or flight" response. This response is characterized by increased heart rate, increased muscle tension, and increased metabolic activity, which are all important aspects of responding to a stressor. The stress response is regulated by CRF and vasopressin neurons in the hypothalamus and noradrenergic neurons in the locus ceruleus (Chrousos, 1992) In addition, a negative feedback system is in place which prevents ACTH secretion when glucocorticoids rise above a threshold concentration. This negative feedback loop is vital, because long-term activation of the HPA axis can result in multiple negative health risks.

Stress Biomarkers and Ethanol

A) Corticotropin-releasing factor

Corticotropin-releasing factor (CRF) is a 41-amino acid chain peptide hormone that regulates the stress response through activation of the sympathetic nervous system, in the central nervous system and periphery (Vale et al, 1981). CRF receptors can we found throughout the central nervous system, but are found at the highest density in the paraventricular nucleus (PVN) of the hypothalamus which projects mostly to the median eminence (Bloom et al, 1982). Intra-PVN jections of glucocorticoids inhibit ACTH release, which indicates that a glucocorticoid negative feedback system acts in the stress circuit (Herman & Cullinan, 1997). Immunohistochemistry studies have identified an increased prominence of CRF neurons in regions of the median forebrain bundle connection to the reticular formation, including the amygdala, bed nucleus of the stria terminalis, and the lateral hypothalamus. CRF mRNA has also detected in the cerebral cortex, and hippocampus, but not the cerebellum (De Souza & Nemeroff, 1989). Figure 1 provides further detail showing additional locations of CRF and stress response

circuitry in the CNS.

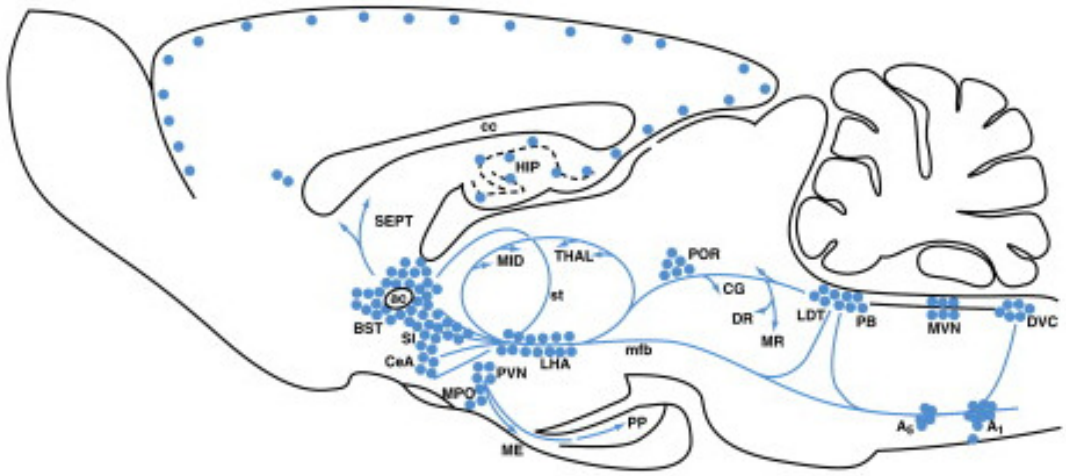

Figure 1. Anatomical areas of action of corticotropin-releasing factor in the brain (Koob, 2008).

CRF receptors are G protein-coupled receptors that are activated by CRF (K_d= 150pM) and other peptides with similar properties, such as urotension and sauvagine, found in fish and frogs respectively (Lovenberg, 1995). In humans and mice, urocortin serves as a ligand for the CRF receptor and CRF- binding protein is an endogenous antagonist to the receptor (Lewis et al, 2001). CRF binds to CRF receptor subtype 1 and 2 (CRF-R1, CRF-R2), which has two splice variants, alpha and beta. CRF-R1 can be found in the pituitary, gonads, and the skin. While CRF-R2 (alpha) is found exclusively in the brain, CRF-R2(beta) can be found in cardiac muscle, epididymis, and gastrointestinal (GI) tract in addition to the brain (Lovenberg, 1995). CRF has been implicated as key aspect of how the body responds to ethanol.

Acute and chronic ethanol exposure has differential effects on the magnitude of CRF release from the hypothalamus. While acute administration (i.p.) of ethanol to male rats increased plasma ACTH and CORT levels, chronically exposing rats to an ethanol vapor

chamber for a week resulted in depressed CRF release (Rivier et al, 1984). The pulse frequency of hypothalamic CRF from rats with a prior history of ethanol consumption was greater than that of ethanol-naïve rats (Redei et al, 1988). Also, these hypothalamic slices that underwent acute ethanol exposure in vitro showed less CRF release than slices that had been previously exposed, suggesting a cellular tolerance to the ethanol (Redei et al, 1988). These differences in acute and chronic ethanol exposure reflect the differences in the physiological response of minimal ethanol drinkers and alcohol abusers to ethanol.

Selective CRF receptor knockout (KO) mice can also be used to characterize differences between the roles of the receptor subtypes, because they can be breed to lack a select receptor. CRF-R1 KO mice showed a decreased release of stress products, ACTH and corticosterone, after restraint stress, despite having similar basal levels as wild type mice (Smith et al, 1998). On the other hand, CRF-R2 KO mice showed a potentiated elevation in ACTH and CORT following restraint stress, suggesting that the two receptor subtypes may play opposite roles during HPA axis activation (Bale et al, 2000; Coste et al, 2000). This point is further supported by studies with CRF-R1/2 KO mice, which show diminished ACTH and CORT in the bloodstream (Priel et al, 2001). The characterization of the stress response of CRF-R1 KO provides initial support for why a CRF-R1 antagonist could be used to reduce social stress-induced behavioral changes.

B) Corticosterone

Corticosterone (CORT) is a corticosteroid hormone secreted from the adrenal gland of vertebrates. During the fight or flight response to a stressful stimuli, rodents produce glucocorticoids, primarily corticosterone, in order to increase their energy. This excess energy, in the form of glucose sugar, allows for more physical activity and gives the rodent a physiological

advantage. Glucocorticoids have also been shown to suppress the immune system by reducing the activity of helper T-cells, which makes the body more susceptible for infection. CORT also works as an anti-inflammatory agent by lowering histamine release. CORT levels can fluctuate over the course of the day as a function of circadian rhythms and environment but can range from about 10ng/ml (morning) to 200ng/ml (late afternoon) for male rats. CORT levels for male mice range from about 50-160ng/ml (Alpco- Mouse and Rat Corticosterone ELISA).

Corticosterone, or cortisol in humans, is the gold standard biomarker for HPA axis activation and is associated with stress and anxiety behaviors in rodents. Anxiety can be tested in rodents by using open field and elevated plus maze tests and analyzing time spent in high-anxiety and low-anxiety regions (Lister, 1987). These tests are commonly used to test the anxiolytic properties of pharmaceutical and experimental compounds (Prut & Belzung, 2003). There was a strong correlation between CORT increases caused by maternal separation in Spraque-Dawley rats and anxiety behavior shown in open field and elevated plus maze tests (Vallée et al, 1997). Pharmacological manipulation of CORT can also be used, which is beneficial because it avoids the variability in the studies that use a stressor to increase CORT. Chronic elevation of CORT in the bloodstream for 14 days, due to implantation of a slow release CORT pellet, resulted in anxiety-like behavior in CD1 mice (Murray et al, 2008). On the other hand, not all studies have shown similar results as repeated daily injection of CORT (40mg/kg, s.c.) for 3 weeks had no effect on the open field anxiety behavior (Gregus et al, 2005). The amygdala has been shown to be a site for CORT action in the CNS, because anxiety behavior in male Wistar rats elevated following CORT micropellets implantation in the central amygdala (Shepard et al, 2000). In addition, acute administration of CORT was sufficient to significantly

increase dendritic spine growth in the basolateral amygdala (Mitra & Sapolsky, 2008), suggesting that a single exposure to stress can cause major physical changes to neuron growth (Mitra & Sapolsky, 2008). Chronic, daily injections of corticosterone have been shown to result in the loss of pyramidal cells from hippocampal CA3 regions in rats (Sapolsky et al, 1985). The interaction between CRF and ethanol is believed to alter the CORT release triggered by stress.

Prior exposure to ethanol has been shown to have an effect on corticosterone levels. In rats, a dose-dependent increase in blood corticosterone occurred following i.p. injections of ethanol (0.5-4g/kg) (Ellis, 1966). This phenomenon was also exhibited in inbred C57Bl/6J and DBA/2J mice (Kakihana et al, 1968). An ethanol injection (i.p.) of 1.6g/kg increased plasma CORT in both strains but 0.8g/kg only increased CORT in DBA/2J mice, showing that ethanol dose and mouse genetic strain play a major role in adrenal cortex stimulation. When the pituitary gland was removed prior to ethanol injections, the drug had no effect on CORT levels, leading to the conclusion that ethanol causes increased release of ACTH from the pituitary (Ellis, 1966). Sex differences in CORT response to ethanol exist, as females displayed higher ACTH and CORT following 0.2-1.8g/kg i.p. injections (Rivier, 2006). Voluntary consumption of ethanol by mice had similar effects on CORT levels, as elevated CORT was maintained for about a week (Tabakoff et al, 1978). It is hypothesized that this elevated CORT in response to ethanol may drive continued ethanol consumption and dependence.

Increased concentrations of corticosterone are associated with increased ethanol drinking, possibly due to the interaction between the reinforcing effects of alcohol and corticosterone (Becker and Lopez, 2011). Adrenalectomized (ADX) male Wistar rats, which could not produce CORT, showed a significant decrease in intake of 6% ethanol compared to their baseline (Fahlke

et al, 1996). When their corticosteroids were restored with intracerebroventricular (i.c.v.) infusion of corticosterone, ethanol drinking returned to their baseline measurements. An additional study showed that ADX rats that underwent one bottle ethanol drinking procedure followed by 3 weeks of ethanol vapor chamber procedure showed significantly lower voluntary 10% ethanol consumption than sham-operated (SHAM) rats (Lamblin and De Witte, 1996). Oral administration of corticosterone, but not hydrocortisone, to the ADX rats increased ethanol intake to that of the SHAM rats (Lamblin & De Witte, 1996). Microinjections of CORT into the ventral striatum of male Wistar rats decreased 6% ethanol drinking, but this effect was not seen when microinjections were directed at the septum, hippocampus, or thalamus (Fahlke & Hansen, 1999). This further research provides evidence that the effects of CORT are locally specific in the central nervous system.

CRF-Antagonists and Alcohol

The focus of the current study is the pharmacological blockade of CRF-R1 with systemic injections of a CRF-R1 antagonist. CRF has been implicated as a vital part of the onset and maintenance of alcohol abuse (Heilig & Koob, 2007). Evidence suggests that CRF may mediate the transition from heavy drinking to alcohol dependence that is sustained by negative reinforcement. The stress response is believed to be activated when an alcoholic experiences withdrawal from alcohol and negative emotional states occur, such as anxiety (Koob & Le Moal, 1997). The mesolimbic dopamine (DA) pathway originates at the VTA, where CRF-R1 is localized, and projects to the nucleus accumbens, hippocampus, and amygdala (Sauvage & Steckler, 2001). The density of CRF neurons that modulate DA neurons in regions of this addiction and reward pathway serves as the rational for targeting CRF-R1.

Administration (i.p.) of CRF-R1 antagonists, antalarmin, MJL-1-109-2, and R121919, significantly decreased the ethanol intake of rats that became ethanol dependent following daily exposure to ethanol vapor (Rimondini et al, 2003). While these antagonists decreased self-administration of ethanol by dependent rats two hours into withdrawal, they had no effect on the drinking behavior of non-dependent rats. Similarly, LWH-63, as well as previously mentioned MJL-1-109-2 and antalarmin, decreased the elevated ethanol drinking shown by Sardinian rats that had a high ethanol preference (Sabino et al, 2006). Recent studies have shown that CRF-R1 antagonists CP-154,526 decreased voluntary ethanol intake of C57BL/6J with either intermittent or continuous access to 20% ethanol (Hwa et al, 2013). Verucerfont and pexacerfont (GSK561679 and BMS-562086 respectively) are CRF-R1 antagonists that proved to be effective at decreasing ethanol intake in preclinical trials and have been advanced to Phase-II clinical trials (Franck & Jayaram-Lindstrom, 2013).

Targeting CRF-R1 has been shown to be an effective treatment for the prevention of stress-induced cocaine seeking behavior as well. CRF was believed to play a similar role in alcohol and cocaine addiction, because i.c.v. infusion of CRF triggered reinstatement of motivation for these drugs (Brown et al, 2009; Le et al, 2000). Episodically socially defeated rats self-administered intravenous cocaine significantly more than non-stressed rats (Boyson et al, 2011). This effect was blocked when systemic injections (20mg/kg, i.p.) or intra-VTA microinjections (0.3µg/0.5µl/side) were administered prior to each of the four social defeats. Due to the similarities between stress-induced cocaine and alcohol seeking, CRF-R1 antagonist, CP-376,395 was proposed as a possible pharmacotherapy to suppress ethanol drinking.

Objective

The role of social defeat stress on ethanol drinking in CFW mice was investigated due to the interaction between the neural mechanism of ethanol action on the central nervous system and the HPA axis. This relationship was explored through assessing two modes of alcohol addiction behaviors, sensitization to an alcohol challenge and voluntary ethanol consumption. The first goal of this research was to determine if a brief (15 attack bite) or moderate (30 attack bite) would trigger CORT levels differentially and whether they would increase voluntary ethanol drinking. The second aim of the study was to use CRF-R1 antagonist, CP-376,395, to decrease stress-escalated ethanol drinking behavior, based on previous literature showing its effects on drinking behavior. The current study also sought to determine if social defeat stress would alter previously acquired ethanol drinking behaviors. It was hypothesized that socially defeated mice would show a sensitization to an ethanol injection and that they would also consume more ethanol during a continuous access to ethanol procedure. This thesis describes the experiments performed to explore the effect of social defeat stress on ethanol sensitization and consumption, as well as the role of CRF-R1 in this behavior.

Materials and Methods

Animals

Adult male Cartworth Farms Webster (CFW) mice (n=39; Charles River Laboratories, Wilmington, MA, U.S.A) weighed 23-25g upon arrival. Experimental mice were group-housed for one week in groups of 6 in a large polycarbonate cage (48x 26cm x 16cm) with corn cob bedding and unlimited access to standard rodent chow (Purina LabDiet 5001) and tap water for

one week. This allowed mice to habituate to the constant temperature (21 ± 1C), 20% humidity, and light/dark photocycle (lights off at 0700 and lights on at 1900) conditions of the vivarium. The mice were then each housed individually in polycarbonate cages (28 x 17 x 12cm) with *ad libitum* access to water and rodent chow. The guidelines of the "Guide for the Care and Use of Laboratory Animals", and the Institutional Care and Use Committee (IACUC) of Tufts University were approved and followed for all procedures (2011).

Social Defeat Stress

Upon arriving in the vivarium, the male, 'resident' CFW mice were individually pair-housed with a female CFW mouse in a polycarbonate cage (28 x 17 x 12cm). After at least two weeks, residents were assessed for aggression by introducing an 'intruder' male CFW mouse into its home cage for 5 minutes. The number of attack bites by the resident mouse were recorded. This procedure was performed for 10 consecutive days. Mice that were determined to be reliably aggressive (greater than 25 bites) were used as 'resident' stimulus mice to socially defeat the experimental mice.

Experimental mice were randomly assigned to be in the moderate (30 attack bites) stress group, the brief (15 attack bite) stress group, or the non-stressed control group. Mice in the control group were weighed daily, while the mice in the stress group were weighed and then socially defeated for 10 consecutive days (Day 1-10) using the following procedure, which consisted of the instigation, defeat, and threat phases (Miczek et al, 1982). In the instigation phase, intruder mice were placed into a perforated, plastic cage (15cm x 7cm x 7cm) and placed into the home cage of an aggressive 'resident' mouse for 5 minutes. During the defeat phase, the intruder mouse was removed from the perforated cage and placed into the resident's cage

without protection. The defeat phase lasted until one of the following conditions was met first; the intruder had received 30 or 15 bites (depending on experimental condition), 5 minutes of confrontation had occurred, or the intruder showed at least 3 consecutive seconds of defeat posture. After one of the previous conditions were met, the intruders underwent the threat phase it was placed back into the perforated cage in the resident's cage for 5minutes.

Locomotor Sensitization

On Day 20, locomotor activity of the mice was recorded in an open field (53cm x 38cm x 46cm) using EthnoVision tracking software (Version 2.4.19). Mice were given a daily injection of saline during the three days prior to this testing in order to habituate to the intraperitoneal injection. Experimental mice were first placed into the open field and allowed to habituate them for 30 minutes. Mice were then given an injection (i.p.) of saline and locomotor activity was recorded for 15 minutes. In the final phase, mice were given an ethanol challenge (2g/kg, i.p.) and movement was recorded for 45 minutes. This dose and length of time were chosen, because it has been shown to be the dose and within the length of time for ethanol to have its most dramatic stimulant effects (Middaugh et al, 1989).

Ethanol Drinking

20% ethanol solutions (w/v) were prepared by diluting 95% ethyl alcohol (Pharmaco-AAPER, Brookfield, CT) with tap water. At least 2 days before locomotor activity was recorded, mice were given access to 2- 50ml centrifuge tubes (Nalgene) with water. Centrifuge tubes were equipped with no. 5 rubber stoppers and stainless steel nozzles were presented to the mice through the metal wire cage lid. The day following the locomotor activity test (Day 21), mice

were given continuous access to a two-bottle choice between 20% ethanol and water for 3 weeks. Bottles were weighed daily in order to determine ethanol and water consumption. The difference in bottle weight was used to measure the average daily fluid consumption. In order to calculate ethanol intake (grams of ethanol per kilogram of body weight), mice were weighed to the nearest tenth of a gram on days that bottle weights were measured. Ethanol evaporation and spillage due to experimenter bottle weighing was determined by weighing a pair of ethanol and water bottles that were held on a cage without an animal. The fluid "drip" measurements were subtracted from the daily ethanol and water bottles weights to calculate the volume of fluid consumption

Systemic Injections of CRF-R1 antagonist and ethanol drinking

Intraperitoneal injections of CP-376,395 (N-[1-Ethylpropyl]-3,6-dimethyl-2-[2,4,6-trimethylphenoxy]-4-pyridinamine hydrochloride), purchased from Tocris Biosciences (Ellisville, MO, U.S.A), were performed. Sterile, physiological saline (Baxter) was used as the vehicle to prepare 1.0, 1.7, and 3.0 mg/ml solutions of CP-376,395. After 3 weeks of continuous access to both a 20% ethanol and water, doses of CP-376,395 (10, 17, 30mg/kg or saline) were administered to the mice. CP-376,395 was chosen for its solubility and its high potency and specificity for CRF-R1 (K_i =12 nM for CRF-R1 and K_i >10000 nM for CRF-R2) (Tocris Biosciences). Ethanol and water bottles were available to mice 15-20 minutes after drug administration and bottles were weighed 2, 4, and 24 hours after access was given. There was at least one day between each drug testing day to ensure that mice returned to baseline fluid drinking levels before the next test day.

Corticosterone Measurements

In experiment 1, a disposable lancet was used for submandibular bleeding 20 minutes after the start of the 'defeat' phase of the social defeat stress procedure. Samples were centrifuged for 10 minutes at 3,000 revolutions per minute and blood plasma was extracted. A Corticosterone Enzyme Immunoassay Kit (Arbor Assays, Ann Arbor, Michigan) was used to analyze the samples for corticosterone.

Experiment 1

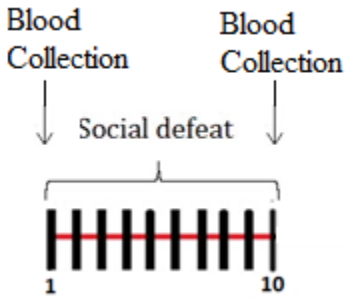

Figure 2. Experiment 1 experimental design

Mice were handled daily for the 3 days prior to the first day of social defeat stress. Mice then underwent 10 consecutive days of either the moderate of brief social defeat stress. On the first (Day 1) and last (Day 10) day of stress, blood was collected 20 minutes after the beginning of the 'defeat' phase. Non-stressed controls were weighed daily during the 10 day protocol and blood was also collected on Day 1 and 10. Blood plasma was later analyzed for corticosterone levels using the Corticosterone Enzyme Immunoassay Kit.

Experiment 2

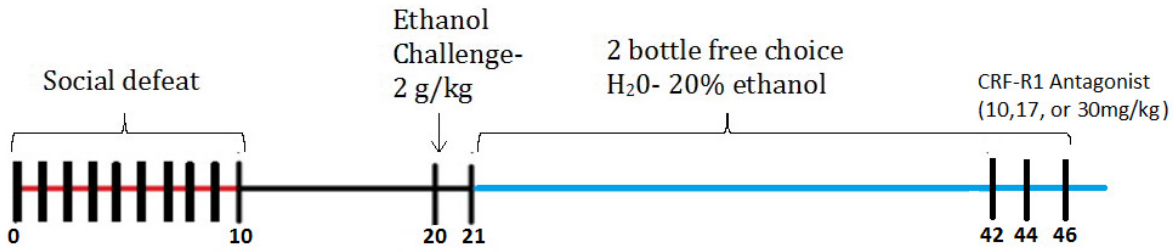

Figure 3. Experiment 2 experimental design

During experiment 2, mice underwent 10 consecutive days of social defeat stress, as previously described. On day 20, mice were tested for locomotor activity following an ethanol challenge. For the next 4 weeks, mice were given continuous access to both a bottle of 20% ethanol and water. During the 4th week, mice were administered differential doses of CP-376,395 and ethanol consumption after 2, 4 , and 24hrs was analyzed.

Experiment 3

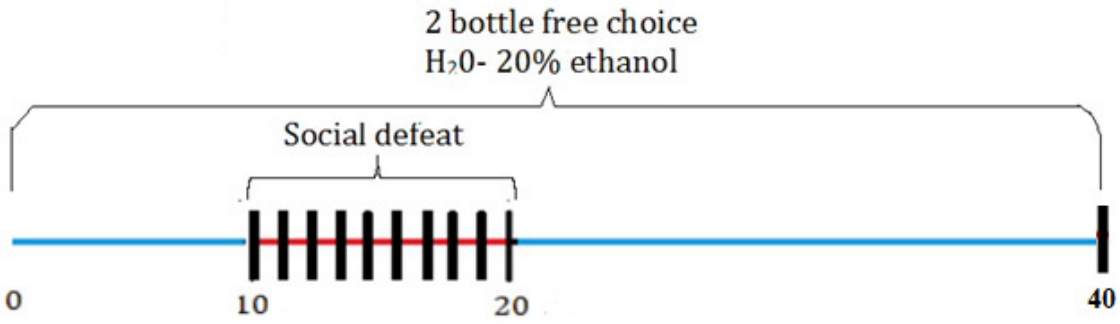

Figure 4. Experiment 3 experimental design

During experiment 3, mice were given access to a 20% ethanol bottle and a water bottle during the duration of the experiment. Baseline ethanol and water drinking established during the first 10 days of the experiment. Mice were divided between the stress and non-stressed group based on their baseline ethanol drinking. In this experiment, mice were socially defeated on

consecutive days during days 10 to 20. Fluid intake was measured on a daily basis during the social defeat procedure.

Statistical Analysis

Descriptive statistics were used to express daily and weekly ethanol consumption and preference as mean ± standard error of the mean (SEM). Ethanol intake (ml) and total volume of fluid consumed (ml) was used to calculated ethanol preference ratio. In Experiment 2 and 3, 5 day ethanol averages were calculated using data from all mice in the group.

SigmaStat 11.0 software (Systat Software, San Jose, California) was used to execute statistical analysis of the data collected. Two-way repeated measures (RM) analysis of variance (ANOVA) were performed to analyze the effect of the two stress procedures on weekly, voluntary ethanol consumption and preference during continuous access to ethanol and water in Experiment 2. Multiple Bonferroni post-hoc t-tests were used to identify significant main effects, as shown by $p<0.05$. The two-way RM ANOVA was also used to determine the effect of the severe stress group on ethanol intake during the 10 day procedure and the three weeks following it. A two-way RM ANOVA was used to indicate that 'high' ethanol drinkers drank significantly more ethanol than 'low' ethanol drinkers before CRF-R1 antagonist was administered. Multiple one-way RM ANOVA was used to determine the effect of systemic CRF-R1 antagonist injections on ethanol drinking of mice following severe social defeat stress.

Results

Experiment 1

Corticosterone Measurements after Social Defeat Stress

The results of Experiment 1 are in progress as blood plasma corticosterone measurements are being analyzed.

Experiment 2

Locomotor Sensitization to Ethanol

While mice in the moderate stress group did not show a change in locomotor activity in response to an ethanol injection, results show a trend of decreased locomotor activity of the controls and severe stress group following the ethanol injection (severe: M=-17.8, SEM=7.5; moderate: M=-0.5, SEM=8.84; control: M= -23.8, SEM=8.89). There was no significant difference in the percent of mice that showed locomotor sensitization to the ethanol challenge (moderate: 17.4%, brief: 21.1%, control: 11.8%). See Figure 5.

There was no correlation between the average daily ethanol intake (g/kg) of either the severe stress, moderate stress, or non-stressed control groups and the change in locomotor activity in response to an ethanol injection.

Weekly Ethanol Intake: Moderate Social Defeat, Brief Social Defeat, and Non-stressed controls

CFW mice were divided into three groups, 30 bite social defeat (moderate, n=23), 15 bite social defeat (brief, n=19), and controls (non-stressed, n=21). Voluntary ethanol and water consumption was compared in these groups during the three weeks of continuous access to ethanol (See Figure 6). These data were also used to calculate ethanol preference (See Figure 7).

The moderate stress group drank more ethanol than both the brief stress group and control group (severe: M= 12.3, SEM= 0.46; moderate: 6.7, SEM= 0.24; control: M=6.66, SEM= 0.21). A two-way repeated measures analysis of variance (ANOVA) revealed that there was a main effect of group [$F(2, 179)= 9.393$, $p< 0.001$] and a main effect of time [$F(3,179)=7.895$, $p<0.001$]. Post-hoc Bonferroni t-tests showed that the ethanol drinking behavior of the moderate stress group was significantly elevated compared to the control group over the course of the 4, 5-day periods of continuous access to ethanol (Days 1-5: $t=2.867$, $p<0.05$; Days 6-10: $t=3.51$, $p<0.01$; Days 11-15: $t=2.74$, $p<0.05$; Days 16-20: $t=2.90$, $p<0.05$). The moderately stress group also had a significantly higher ethanol intake than the brief stress group during the three weeks (Days 1-5: $t=2.61$, $p<0.05$; Days 6-10: $t=2.84$, $p<0.05$; Days 11-15: $t=3.14$, $p<0.01$; Days 16-20: $t=3.27$, $p< 0.01$). There was no difference between the ethanol drinking behavior of the non-stressed controls and the brief stress group. Moderately stressed mice showed heightened ethanol drinking during the final 5 day period compared to the first 5 day period ($t=3.34$, $p<0.01$). This provides evidence for an initial and persistent increase in ethanol consumption among the moderate stress group.

The moderate stress group also demonstrated elevated ethanol preference during the experiment, compared to the brief stress group and non-stressed group. There was a main effect of time [$F(3,179)=3.632$, $p<0.05$] and group [$F(2,179)= 7.916$, $p<0.001$) on ethanol preference during the three weeks of two-bottle free choice as demonstrated by a two way RM ANOVA. Further analysis using Bonferroni post-hoc tests illustrated that weekly ethanol preference average was significantly higher than non-stressed controls during days 6-20, but not the first 5 days (Days 6-10: $t=2.84$, $p<0.05$; Days 11-15: $t=2.80$, $p<0.05$; Days 16-20: $t=3.20$, $p<0.01$).

There was also a significant difference between ethanol preference between the moderately and briefly stressed groups during days 6-10 and 16-20 of drinking, with a trend for days 11-15 (Days 6-10: $t=2.98$, $p=0.01$; Days 11-15: $t=2.38$, $p=0.056$; Days 16-20: $t=3.11$, $p<0.01$). This statistical analysis supports the claim that the moderate stress group had a heightened ethanol preference during days 6-20 of ethanol access. An additional Bonferroni post-hoc t-test revealed that the ethanol preference of the moderate stress group was heightened between the first 5 days and the last 5 days of the experiment ($t=3.29$, $p<0.01$), as ethanol preference increased for this group over time.

Overall, water intake was not altered during the experiment, but it was shown to decrease among the moderate stress group during the third week (See Figure 8). There were no differences in total volume of fluid consumed during the extent of the experiment (See Table 4). There was a main effect of time on water intake [$F(3,178)=7.180$, $p<0.001$], as identified with a two-way RM ANOVA, but there was no main effect of group on water intake. Although there was not a significant difference between the moderate stress, brief stress, and non-stressed group, Bonferroni post-hoc tests showed that there was a decreased water intake between the first and final 5 day period ($t=3.19$, $p=0.01$).

Overall, the weight of mice in the three groups did not significantly differ (moderate stress: $M=33.3$, $SEM= 0.12$; brief stress: $M=33.2$, $SEM= 0.13$; non-stressed: $M=32.2$, $SEM=0.16$). See Figure 9. Moderately stressed mice showed a significant increase in weight between the first two 5 day periods ($t=5.37$, $p<0.001$). Briefly stressed mice showed a delayed weight increase as they did not show increased weight until Days 11-15 ($t=3.48$, $p <0.01$). Both stressed groups showed earlier weight gains than non-stressed controls, which did not exhibit

weight gain until Days 16-20 ($t=2.75$, $p<0.05$). There was no main effect of group on daily mice weight, but there was a main effect of time on mice weight [$F(3,179)=3.55$, $p<0.001$).There was also no correlation between average weight and ethanol intake during the 3 weeks of ethanol access.

Systemic CRF-R1 Antagonist: Effect on High and low ethanol drinkers

After it was shown that the 30 bite social defeat procedure increased voluntary ethanol consumption, the role of CRF-R1 was explored by administering a CRF-R1 antagonist, CP-376,395, to the CFW mice (n=34). Ethanol and water intake was recorded 2, 4, and 24hrs after the administration of the drug (10, 17, and 30mg/kg). Stressed mice were divided into 2 groups, high and low ethanol drinkers, based on their ethanol consumption during the 3 weeks of continuous access to ethanol. High drinkers were defined as mice that drank more than 10g/kg ethanol for the majority (>50%) of days and low drinkers were mice that drank less than 10g/kg of ethanol for less than 50% of days (See Figure 10).

CP-376, 395 was shown to significantly decrease the ethanol intake (g/kg) of high drinkers in 2 and 4hr [$F(3,24)= 4.759$, $p= 0.01$; $F(3,24)= 6.006$, $p<0.01$]. See Figure 11. Post-hoc Bonferroni t-tests further showed that only the highest dose of the drug (30mg/kg) decreased ethanol drinking at the two time points [2hr: $t=3.32$, $p<0.05$; 4hr: 3.73, $p<0.01$]. Lower doses of CP-376,395 (10 and 17mg/kg) had no effect on the ethanol intake of the CFW mice at either 2, 4, or 24hr following administration. None of the drug doses affected water drinking (ml) in 2, 4, or 24hr of high ethanol drinking mice. See Table 5.

Experiment 3

Effect of Moderate Social Defeat on Previously Acquired Ethanol Drinking

The moderate social defeat stress protocol increased ethanol intake and preference ratio in a delayed manner (See Figure 12). The baseline average for all mice during the 10 days before the start of social defeat stress was 6.77 g/kg. The baseline for the two groups were not significantly different (Stress: M= 7.42, SEM= 0.59; Non-stressed: M= 6.02, SEM= 0.52). There was a main effect of time of ethanol intake during the experiment [$F(6,144)= 5.670$, $p<0.001$]. Holm-shidak post-hoc tests revealed a trend of decreased ethanol drinking during the first 5 days of social defeat compared to baseline ($t=2.582$, $p=0.063$). Holm-shidak tests also indicated a trend towards increased ethanol drinking during the last 5 days of ethanol access compared to baseline ($t=2.542$, $p=0.059$).

Socially defeated mice showed a delayed increase in ethanol preference ratio. A main effect of time on ethanol preference ratio was present [$F(6,144)=4.301$, $p<0.001$]. Holm-shidak post-hoc tests showed that socially defeated mice have significantly higher ethanol intake during the last 5 days of ethanol access compared to ethanol intake during the last 5 days of the social defeat procedure ($t=3.288$, $p<0.05$).

Discussion

Main Findings

The results of the current study show that the moderate (30 attack bites) social defeat stress procedure prior to ethanol access leads to an increased, persistent voluntary ethanol intake and ethanol preference compared to non-stressed controls and the brief (15 attack bites) social defeat stress procedure. The brief social stress procedure did not induce ethanol consumption changes, compared to non-stressed controls. When ethanol access is given prior to the moderate

social defeat stress protocol, there is a delayed increase in ethanol intake and preference between stressed and non-stressed mice. CP-376,395 decreased ethanol intake of the high ethanol drinkers in a dose-dependent manner during the initial 2 hours compared to the low ethanol drinkers; after 4 hours the drug effect was only observed at the highest dose. Current experiments provide evidence that CRF-R1 may play a critical role in mediating stress-induced ethanol consumption.

Experiment 1: Social Defeat Stress and Blood Plasma Corticosterone

Blood plasma is currently being analyzed for corticosterone levels following both the 1st and 10th day of social defeat stress.

Experiment 2: Role of CRF-R1 on Social Defeat Stress, Alcohol Sensitization and, Voluntary Ethanol Consumption

Alcohol Sensitization

The average daily ethanol intake (g/kg) of neither the moderate stress, brief stress, nor non-stressed control groups predicted the change in locomotor activity in response to an ethanol injection. For the majority of mice, cross sensitization between ethanol injection (i.p.) and stress was not present. It was hypothesized that socially defeated mice would show increased locomotor sensitization to ethanol as a result of the neuroplastic changes from stress that sensitize the mesolimbic pathway involved with reward (Fish et al, 2002). Cross-sensitization was expected to be expressed, because aversive stimuli like stress activate mesocortical and nigrostriatal dopamine pathways and this potentiates dopamine transmission when a rewarding stimulus, such as amphetamine, is administered (Robinson & Berridge, 1993).

Previous studies have suggested that stress has been shown to increase locomotor sensitization and self-administration of addictive psychomotor stimulant drugs, including cocaine and amphetamine (Phillips et al, 1997). Voluntary ethanol consumption did not predict

locomotor sensitization to an ethanol challenge. This was unexpected, because increased locomotor activity in response to ethanol administration has been shown to be genetically linked to high ethanol consumption (Piazza & Le Moal, 1996; Grahame et al, 2000).

Voluntary Ethanol Consumption

CFW mice had increased voluntary ethanol consumption following exposure to a moderate 10-day social defeat procedure with 30 daily attack bites, but not a brief 15 daily attack bite procedure. It is important to note that the average ethanol preference did not exceed 50%, indicating that water was still preferred over the 20% ethanol solution. The results of the current study shed light on parameters of social defeat stress that influence ethanol drinking that are otherwise unclear. The longer duration of time spent in an stressful environment in the moderate stress group may induce more long-lasting neuroplastic changes to CRF neurons in the CNS that make the outbred mice more vulnerable to ethanol drinking behavior. The neuroadaptions caused by moderate stress strengthens over time, while if the moderate stress caused any neuroadaptions at all, the short-term effects deteriorate before any change in ethanol drinking are apparent. A more chronic, severe stress would likely result in a more depression-like state, characterized by anhedonia and reduced ethanol drinking (Nestler et al, 2006; Iniguez et al, 2014). The increase in ethanol drinking during the current experiment could be caused by altered function of CRF neurons following the stress period. These CRF neurons control the action of dopaminergic neurons, which play a major role in the mesolimbic pathway of reward and addiction. The 30 attack bite, but not the less stressful 15 attack bite, protocol resulted in extensive HPA activation and changed the mesolimbic dopamine system which induced heightening ethanol seeking behavior (Miczek et al, 2008).

The proposed mechanism behind this altered behavior in ethanol drinking involves a change in the CRF receptors on dopaminergic neurons in the VTA. Further exploration would be needed to determine if the more intense HPA activation as a result of the moderate social defeat stress protocol increased CRF-R1 receptor density greater than that of the brief social defeat stress procedure. This change would suggest that increased levels of glucocorticoids released following the moderate stress led to long-term neuroplastic changes in the dopaminergic mesolimbic pathway that the propensity for voluntary ethanol consumption.

The current experiments found that the weight of the moderately stressed mice escalated at a higher rate than non-stressed controls. This effect is likely caused by the high caloric value of ethanol, rather than a direct result of stress. The findings contrast to many studies that show that stress decreases body weight in rodents (Rygula et al, 2005). The main difference between body weight changes in the current studies and previous literature is that most previous studies observe weight changes during the stress procedure, while the current study showed long-term weight changes. This may be explained by the corticosterone release from adrenal glands during the 10 day stress response. Intracerebroventricular infusions of corticosterone have been shown to cause body weight increases in underweight rats (Green et al, 1992). In contrast, repeated i.p. injections of differential doses of corticosterone decreased rat weight in a dose-dependent manner (Johnson et al, 2006).

Systemic CRF-R1 Antagonist Injections

The results of the pharmacological manipulation suggest that CRF-R1 plays an important role in the onset of stress-induced ethanol drinking. After exposure to the moderate (30 attack bites) social defeat stress, mice were grouped into high and low ethanol drinking groups based on

their drinking during 3 weeks of access to ethanol and water. High drinkers were shown to have a higher ethanol intake than low drinkers on every day of ethanol access during the 3 weeks of ethanol access. All high drinkers accumulated more ethanol intake over the experiment than low drinkers. This consistency in behavior between the two groups adds to the validity of dividing the CFW mice into groups based on these individual differences. The genetic variability that underlies behavioral differences within outbred strains leads to the rationale for exploring the differential effects of a CRF-R1 antagonist on the behaviors of these two groups.

The CRF-R1 antagonist decreased ethanol drinking in a dose dependent manner for the high drinkers, but had no effect on low drinkers or non-stressed controls drinking. We found that mice that experienced the 30 attack bites, but not the 15 attack bites, during social defeat stress consumed more ethanol than non-stressed controls. CP-376,395 decreased ethanol intake of the high ethanol drinkers in a dose-dependent manner during the initial 2 hours compared to the low ethanol drinkers; after 4 hours the drug effect was only observed at the highest dose.

It is possible that a reduction in ethanol drinking was not seen in the low drinking group after CP-376,395 injections due to a basement effect. Because the mice in this group were already drinking a low amount, ethanol drinking would have to have been almost completely eliminated in order to reveal a significantly decreased ethanol consumption after injections of the CRF-R1 antagonist injections. It is unlikely that only blocking part of the CRF system would lead to the complete inhibition of ethanol intake due to the complex circuitry of neurotransmitters and neuropeptides involved in alcohol drinking behaviors. The modulation of neuropeptide Y and the opioids system have also been shown to play a critical role in alcohol dependence (Phillips et al, 1997; Sparrow et al, 2012). While blocking CRF-R1 receptors

decreased ethanol drinking in high ethanol drinkers, targeting multiple systems may be required to decrease ethanol intake in the low ethanol consumption group.

Results of the current experiment suggest that dysregulation of CRF by social defeat stress causes prolonged changes in the brain that drive excess ethanol intake. The CRF antagonist is believed to decrease ethanol drinking, because it suppresses dopamine-CRF interactions in the mesolimbic reward pathway. Dopaminergic neurons located in the ventral tegmentum area have been shown to increase their firing rate following social defeat stress in rats (Anstrom et al, 2009). Modulation of dopaminergic neuron firing by CRF has been shown in the ventral tegmentum area (Wanat et al, 2008). Evidence of a bidirectional relationship between these two components of stress-induced drug addiction is apparent, because CRF-R1 is required for the process by which dopamine release strengthens glutamatergic synapses in the amygdale (Kash et al, 2008). By blocking CRF-R1 activation, the facilitation of dopaminergic neurons that drive persistent ethanol seeking behavior is prevented.

Experimental 3: Effect of Social Defeat Stress of Ethanol Drinking of mice with history of ethanol drinking

Voluntary Ethanol Drinking

Mice that had prior access to ethanol showed decreased ethanol drinking during the 10 day moderate stress protocol. Following the conclusion of the stress period, there was a delayed increase in ethanol drinking that was only evident towards the end of the ethanol access period. Although Experiment 2 showed that ethanol intake increased immediately in CFW mice with a history of severe social defeat stress, this effect was eliminated when mice were given access to ethanol prior to stress and during the stress procedure. This information displays the importance of the order of which stress occurs in relation to when ethanol drinking is acquired. The results of

the study are inconsistent with previous studies using mice where social defeat stress increased previously acquired ethanol drinking (Sillaber et al, 2002; Croft et al, 2005).

Social defeat stress resulted in a delayed increase in previously acquired ethanol drinking. It is believed that the previous exposure to ethanol prior to social stress suppressed the HPA axis activation. The neuroplastic changes in the brain of mice that drank ethanol prior to stress made the mice resistant to the social defeat stress. This decrease in the stress response attenuated voluntary ethanol consumption and prevented an initial rise in ethanol drinking. Similar results were found in a rat model of PTSD in which inescapable foot shocks increased ethanol drinking, but had no effect on ethanol drinking if the behavior had already been established (Meyer et al, 2013). A possible confound in comparing this experiment to Experiment 2 is the duration of time spent single-housed. In experiment 2, mice were single housed for 20 days before given ethanol access while mice in Experiment 3 were given ethanol access within a day of being single-housed.

Social Defeat Stress and Motivation for Ethanol

Current studies based on the results of this research project have shown that social defeat stress increases motivation for ethanol during an intermittent access to ethanol procedure (IAA, See Figure 13). This experiment was set up in a similar manner are Experiment 2 of the current study, but mice were given daily access to ethanol intermittently throughout the week (Hwa e al, 2011). Mice were placed in an operant conditioning chamber during the first few hours of ethanol access days and completed a nose-poke task for either a reinforcement of 20% ethanol or water. CFW mice that experienced the moderate social defeat protocol worked for

more ethanol reinforcements during a progressive ratio schedule of reinforcements than non-stressed controls. This provides further evidence for a relationship between social stress and ethanol intake that extends beyond voluntary, ethanol consumption.

Limitations

Follow-up studies using an IAA procedure would be useful for alleviating the issues of using a continuous access to ethanol procedure for pharmacological manipulation studies. During the continuous access procedure, the amount of ethanol consumed prior to drug administration is unknown. Although the drugs were given 2-3hours into the dark cycle, when mice have been shown to consume the most ethanol, individual differences in behavior exist. In an IAA procedure, researchers can control for this, because all mice were water restriced for the 24hours prior to drug administration. It is important to note that it would need to be established that social defeat stress increases ethanol consumption during this procedure before pharmacological manipulations be performed.

Although precautions were taken to ensure that each daily social defeat experience was as similar as possible, slight individual differences occurred. Only the most aggressive 'resident' CFW mice were used as stressors, but their daily variation in aggression may have created a difference in the stress experiences, because some intruders were attacked 30 times in 5 minutes, while some were reached the attack threshold within 1 minute. The 5 minute maximum for the 'defeat' phase of the procedure was determined to be a reasonable duration for resident mice to attack the intruder mice 30 times.

Future Studies

While the current study uses a continuous access to ethanol procedure in order to show voluntary ethanol consumption, future studies could use a different model of ethanol drinking. Using an intermittent access to alcohol (IAA) procedure may result in murine intoxication and clear signs of withdrawal by analyzing handle-induced convulsions during a withdrawal period (Rhodes et al, 2005; Goldstein, 1983). The IAA procedure has been how to produce increased ethanol drinking and preference than mice with continuous access (Hwa et al, 2011). In addition, C57BL/6J mice exhibited blood ethanol concentrations above 100mg/dl and showed significant withdrawal behavior during the IAA procedure. It would allow for a more accurate analysis of pharmacological manipulations which will be further explained in the next paragraph. A drawback to this procedure is that the stress of withdrawal that works to maintains high drinking behavior due to the "kindling effect" may interact with the social defeat stress. Ethanol withdrawal has been shown to be a stressful event as indicated by increased levels of CRF (Pich et al, 1995).

A further understanding of the compulsive or impulsive nature of alcohol drinking better characterize stress-induced ethanol drinking behavior. Compulsive alcohol drinking, defined by irresistible urges, could be explored by the use of a schedule of ethanol that is limited (Caron & Pruett, 1996). Compulsivity could also be tested using a paradigm where mice must endure an electric foot shock in order to receive an ethanol reinforcement. This would distinguish whether or not the drive for ethanol is worthy of receiving an aversive stimulus. Impulsivity, or novelty-seeking and risk-taking behavior, is a characteristic of drug taking behavior in humans (Kreek et al, 2005). Impulsivity can be tested using a variety of tests including delayed reward tasks, which

indicate self-control (Bent & Isles, 2011). Previous literature suggests that the impulsivity shown by clinical populations of drug users could be due to prior exposure to stress (Cleck et al, 2008).

Additional research studies would further investigate the role of CRF-R1 in stress-induced ethanol drinking, while further exploring CRF-R2 and CRF-BP. If Antalarmin, another CRF-R1 antagonist, decreased stress-induced high ethanol drinking similar to that of CP-376,395, it would strengthen the argument that CRF-R1 plays a major role in mediating the relationship between stress and ethanol drinking. Considering the differential roles of CRF-R1 and CRF-R2 shown by genetic knockout studies, one might expect CRF-R2 to have opposite effects on ethanol drinking (Coste et al, 2000). CRF-BP can be blocked by applying a CRF fragment that will compete for the CRF binding site and prevent CRF from acting on CRF-BP. CRF-BP has been showed to be necessary for the potentiation of NMDAR activity in dopaminergic neurons in the VTA that is characteristic neuronal response to addiction and stress (Ungless et al, 2003). Microinfusions of CRF-BP into the VTA reduced footshock-induced reinstatement of cocaine seeking in mice, further suggesting a major role of CRF-BP in stress and drug addiction (Wang et al, 2007).

Further understanding of relationship between CRF pathways in the brain and ethanol drinking could be explored by the use of optogenetic techniques. It is possible that recurrent stimulation of CRF neurons in areas of stress neurocircuitry, including the central amygdala and ventral tegmentum area, could induce the long-term behavioral effects on ethanol drinking caused by the moderate social defeat stress procedure (Cui et al, 2013). Tonic activation of dopaminergic neurons in the ventral tegmentum area have already been shown to decrease ethanol intake in rats, which reveals the success of using optogenetics to target specific anatomical sites related to drug addiction. The strategic use of this technique would allow

researchers to gain further insight on the neurobiological mechanisms that underlie stress-induced ethanol drinking by targeting overlaying pathways of stress and reward.

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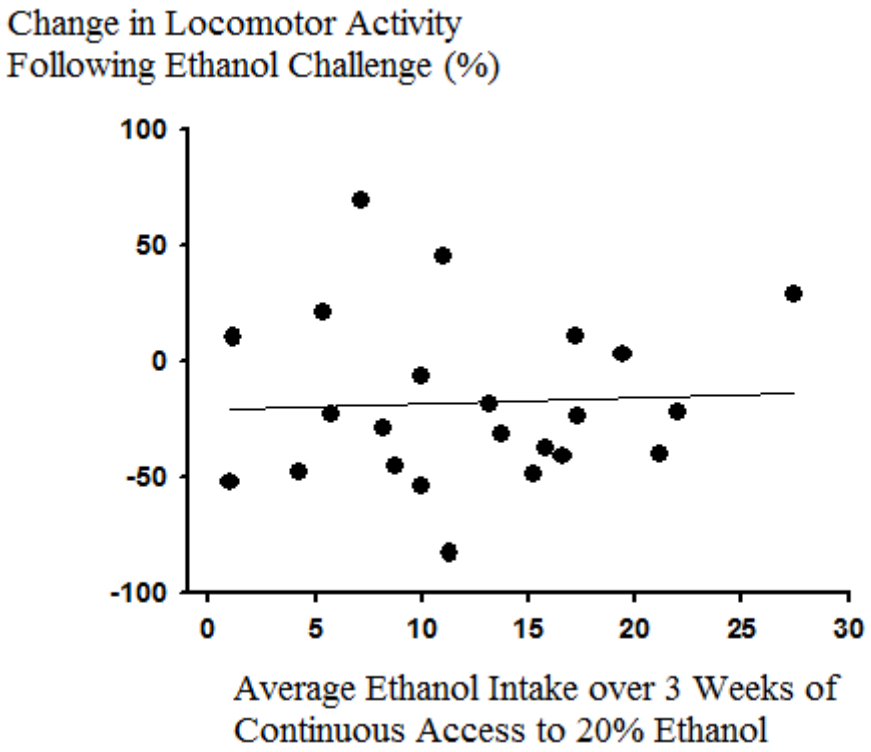
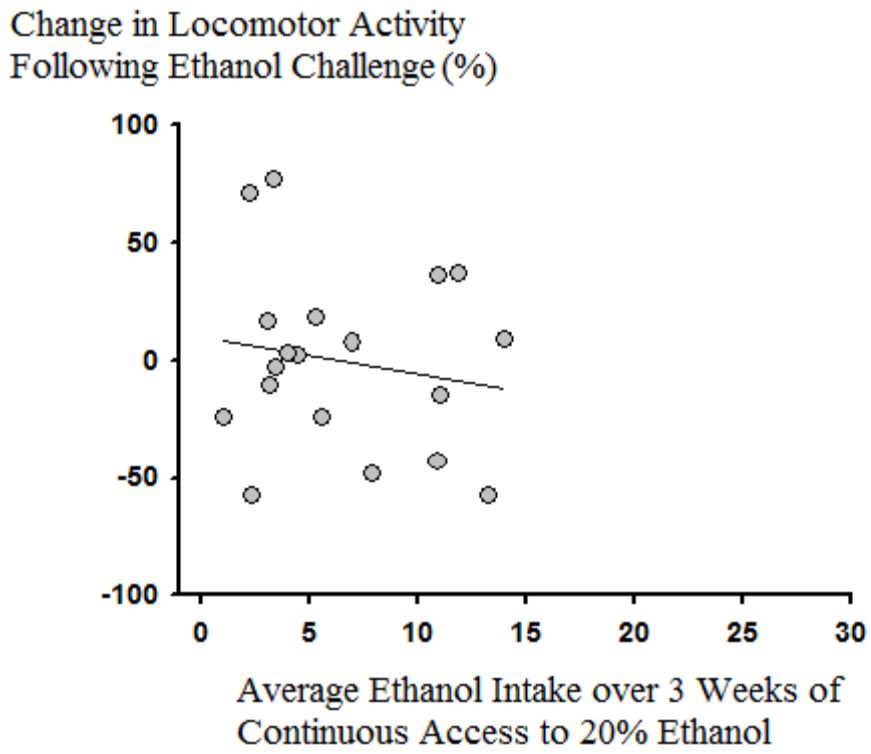
Figure 5. A) Relationship between average, daily ethanol intake (g/kg) during 3 weeks of ethanol access and locomotor activity change in response to ethanol challenge (%) of moderate stress group (n=23). B) Relationship between average, daily ethanol intake (g/kg) during 3 weeks of ethanol access and locomotor activity change in response to ethanol challenge (%) of brief stress group (n=19) . C) Relationship between average, daily ethanol intake (g/kg) during 3 weeks of ethanol access and locomotor activity change in response to ethanol challenge (%) of non-stress group(n=21).

A)



B)


Change in Locomotor Activity
Following Ethanol Challenge (%)
100
50
0
-50
-100
0
5
10
15
20
25
30
Average Ethanol Intake over 3 Weeks of
Continuous Access to 20% Ethanol

C)

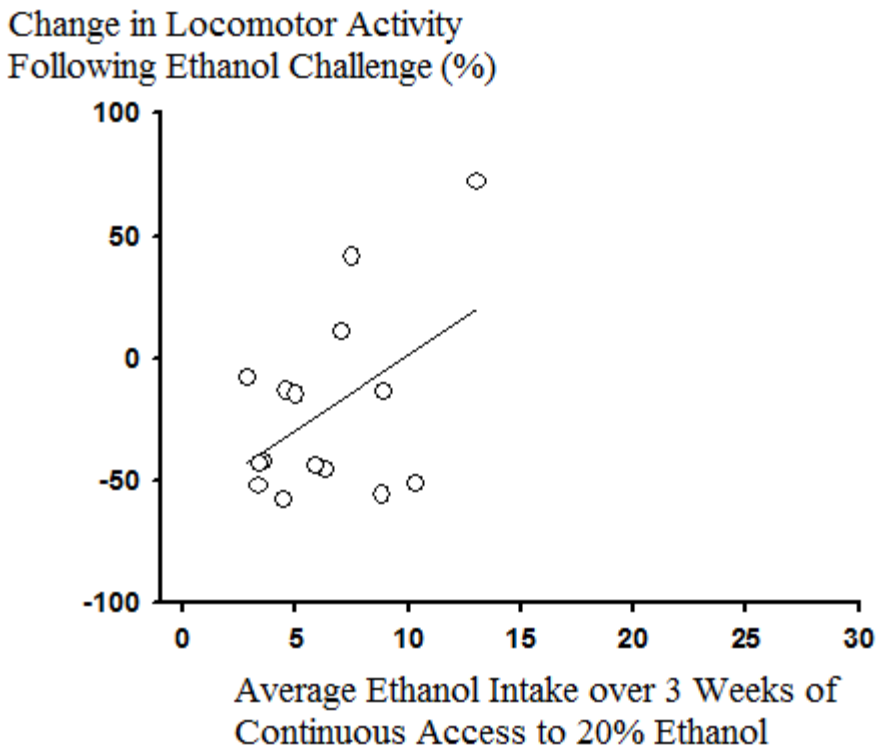
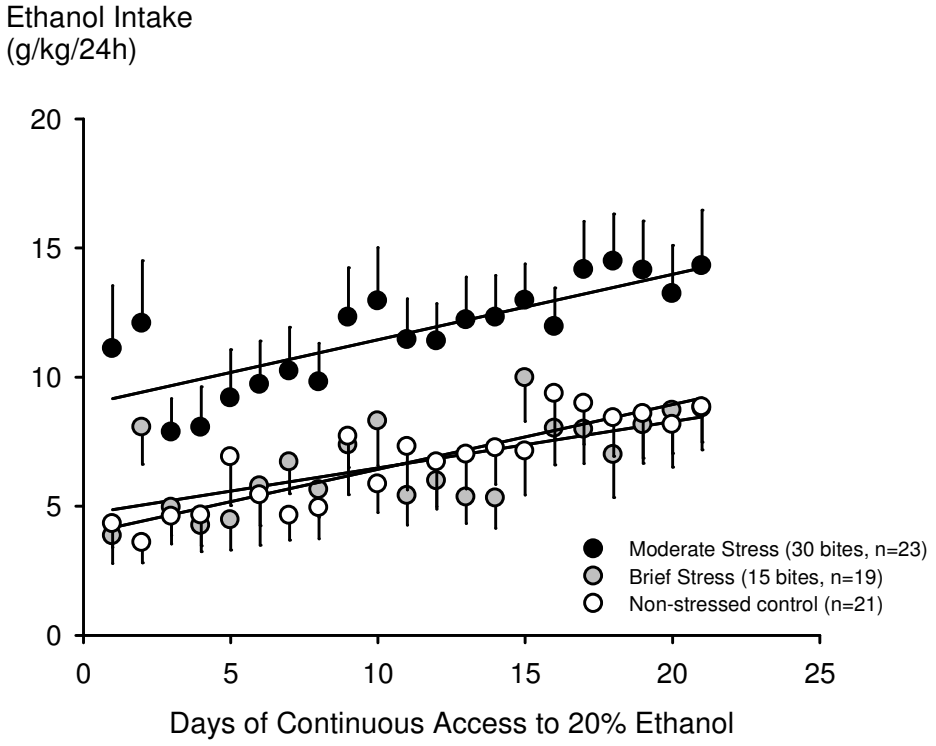

Change in Locomotor Activity
Following Ethanol Challenge (%)
100
50
0
-50
-100
0
5
10
15
20
25
30
Average Ethanol Intake over 3 Weeks of
Continuous Access to 20% Ethanol

Figure 6. A) Daily, voluntary ethanol intake (g/kg) of moderate stress, brief stress, and non-stressed controls during a 3 week period of continuous access to 20% ethanol and water. B)The daily ethanol intake of the moderate, brief, and non-stressed groups over the 3 weeks of continuous access to 20% ethanol are converted into 5 day averages. Error bars indicate the standard error of the mean. Asterisks indicate significant increase in ethanol drinking in the moderate stress group compared to ethanol intake of the brief stress and non-stressed CFW mice during that week ($p<0.05$). Pound signs indicate significant increase in ethanol drinking in the moderate stress group compared to its baseline drinking during the first 5 day period. ($p<0.05$).

A)



B)

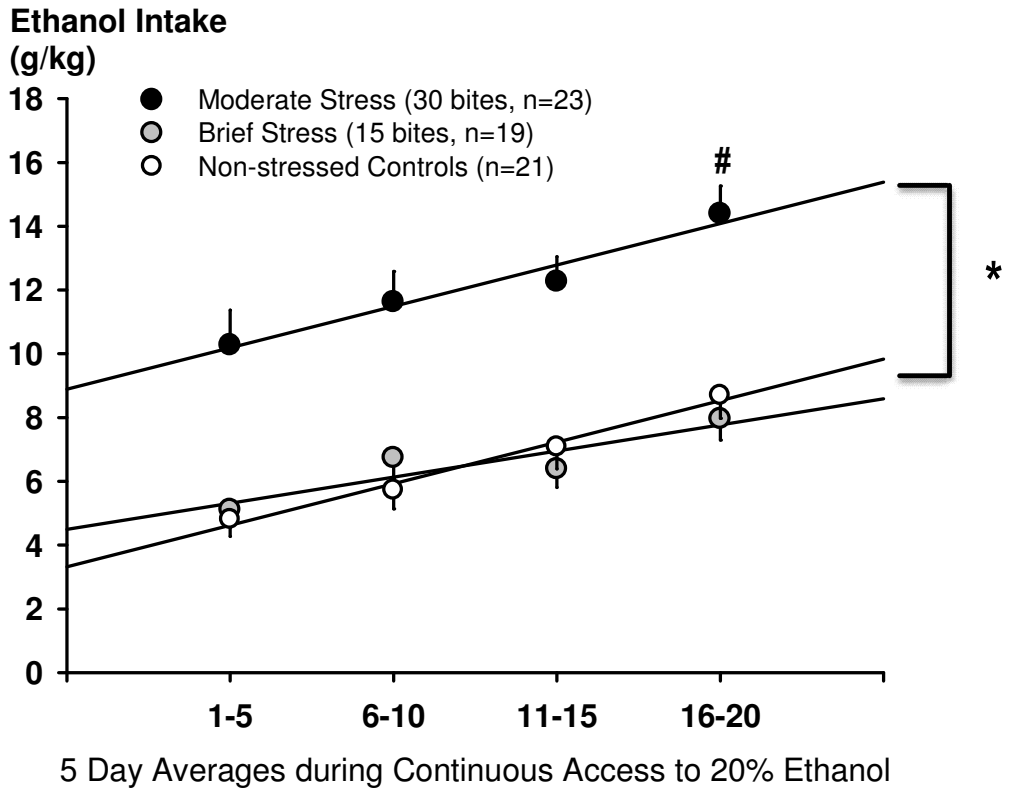
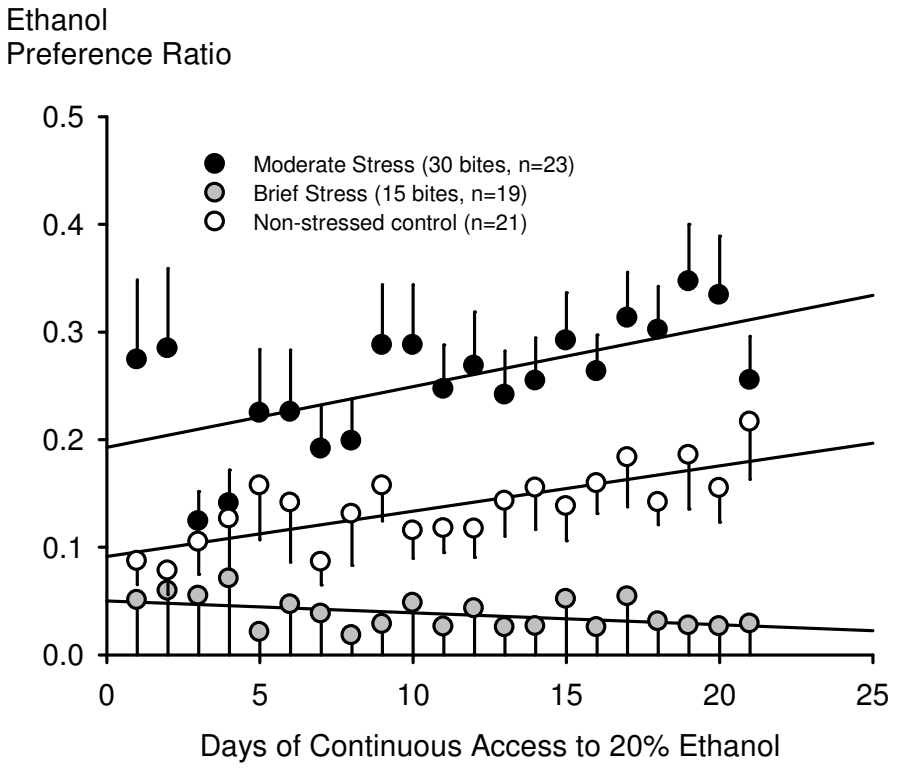

Ethanol Intake
(g/kg)
Moderate Stress (30 bites, n=23)
Brief Stress (15 bites, n=19)
Non-stressed Controls (n=21)
18
16
14
12
10
8
6
4
2
0
#
*
1-5
6-10
11-15
16-20
5 Day Averages during Continuous Access to 20% Ethanol

Figure 7. A) Daily voluntary ethanol preference ratio of moderate stress, brief stress, and non-stressed controls during a 3 week period of continuous access to 20% ethanol and water. B) The daily ethanol preference ratio of the moderate, brief, and non-stressed groups over the 3 weeks of continuous access to 20% ethanol were then converted into 5 day averages. Error bars indicate standard error of the mean. Asterisks indicate significant increase in ethanol preference in the moderate stress group compared to ethanol preference of the brief stress and non-stressed CFW mice during that week ($p<0.05$). Asteriks in parenthesis indicate the difference was close to statistical significance ($p<0.06$). Pound signs indicate significant increase in ethanol drinking in the moderate stress group compared to its baseline drinking during the first 5 day period. ($p<0.05$).

A)



B)

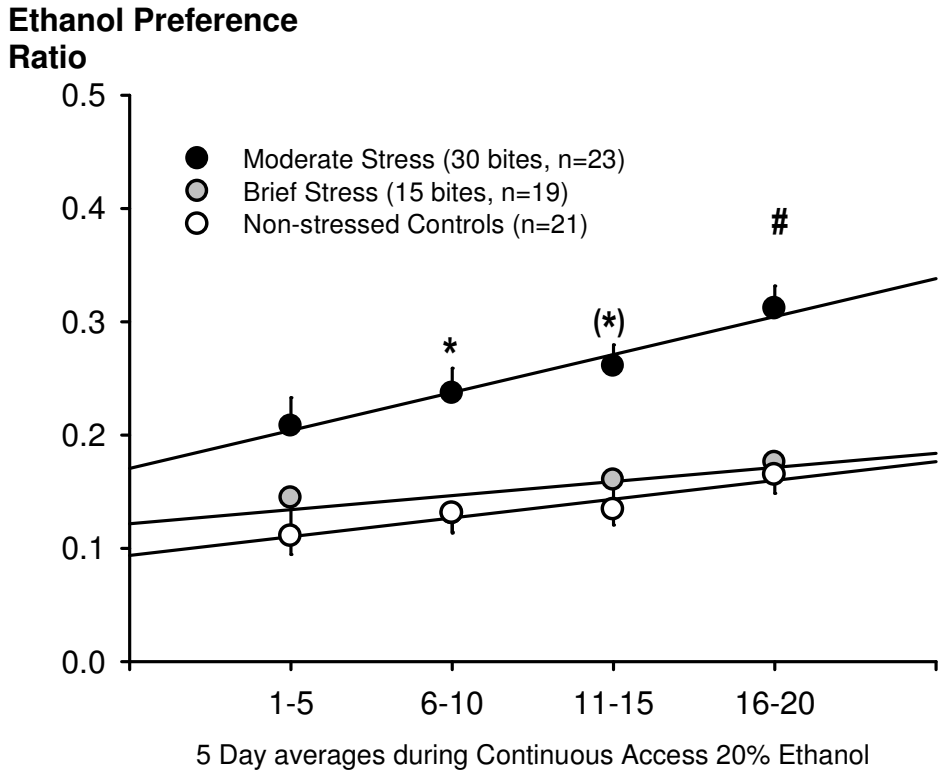

Ethanol Preference Ratio
0.5
0.4
0.3
0.2
0.1
0.0
Moderate Stress (30 bites, n=23)
Brief Stress (15 bites, n=19)
Non-stressed Controls (n=21)
*
(*)
#
1-5
6-10
11-15
16-20
5 Day averages during Continuous Access 20% Ethanol

Figure 8. The daily water intake (ml) of the moderate, brief, and non-stressed groups over the 3 weeks of continuous access to 20% ethanol were converted into 5 day averages. Error bars indicate standard error of the mean.

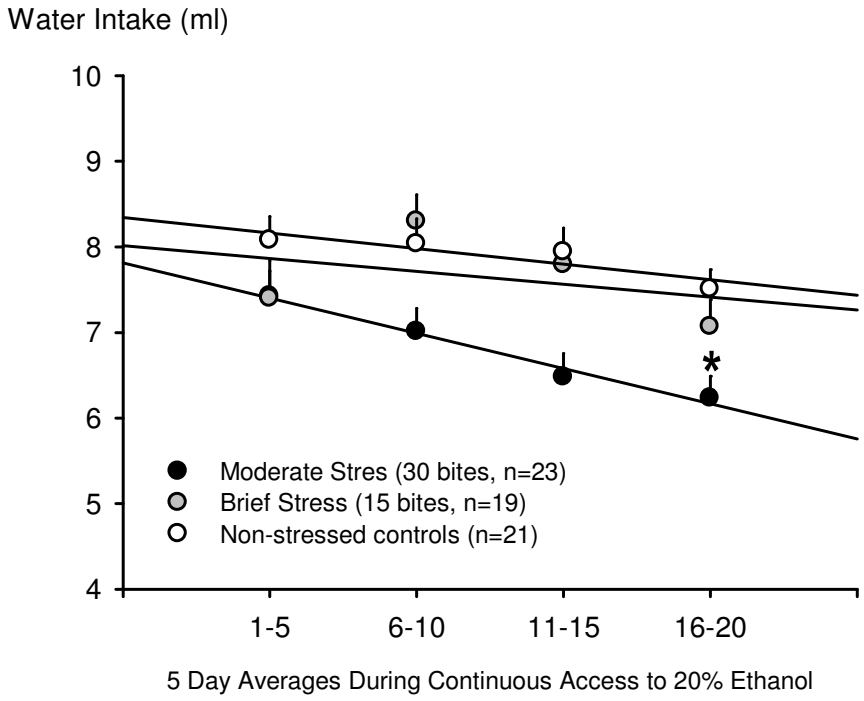


Table 4. Effects of Stress on Total Volume of Fluid Consumed

Stress Group	Day	Total Volume (ml)
Moderate Stress	1-5	9.1±0.18
	6-10	8.9±0.2
	11-15	8.4±0.26
	16-20	8.5±0.23
Brief Stress	1-5	8.7± 0.33
	6-10	9.5± 0.24
	11-15	9.2± 0.24
	16-20	9.2± 0.27
Non-stressed	1-5	8.8± 0.27
controls	6-10	9.0±0.27
	11-15	9.1±0.28
	16-20	8.8±0.23

Figure 9. The daily mouse body mass (g) of the moderate, brief, and non-stressed groups over the 3 weeks of continuous access to 20% ethanol were converted into 5 day averages. Error bars indicate standard error of the mean.

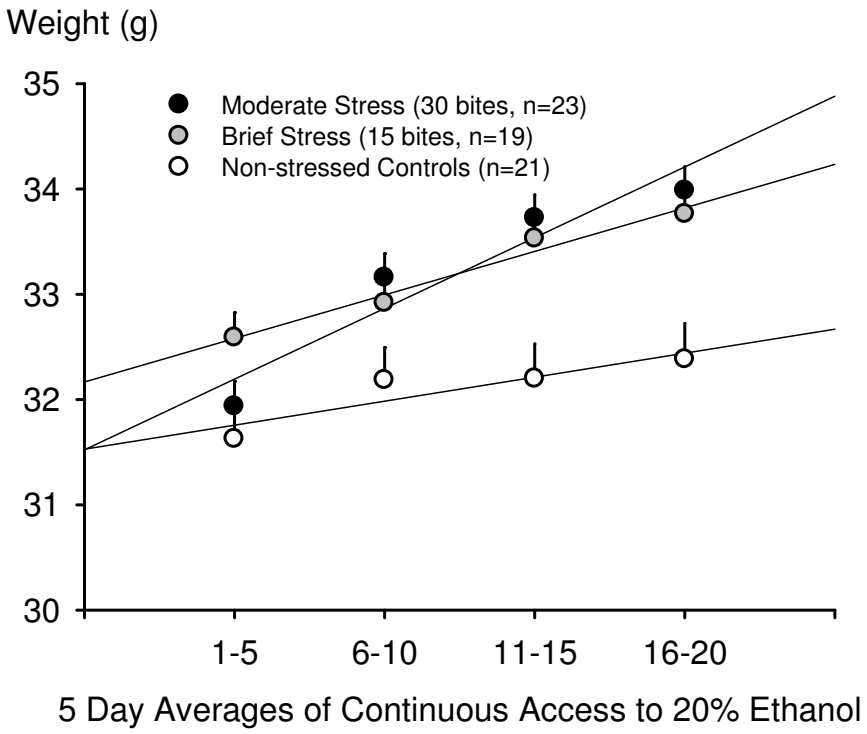


Figure 10. The daily ethanol intake of high and low ethanol drinkers, based on their ethanol drinking during the 3 weeks of continuous access to ethanol, as well as control group are shown. Error bars indicate standard error of the mean. An asterisk denotes a significant difference between group, based on daily ethanol intake ($p<0.05$).

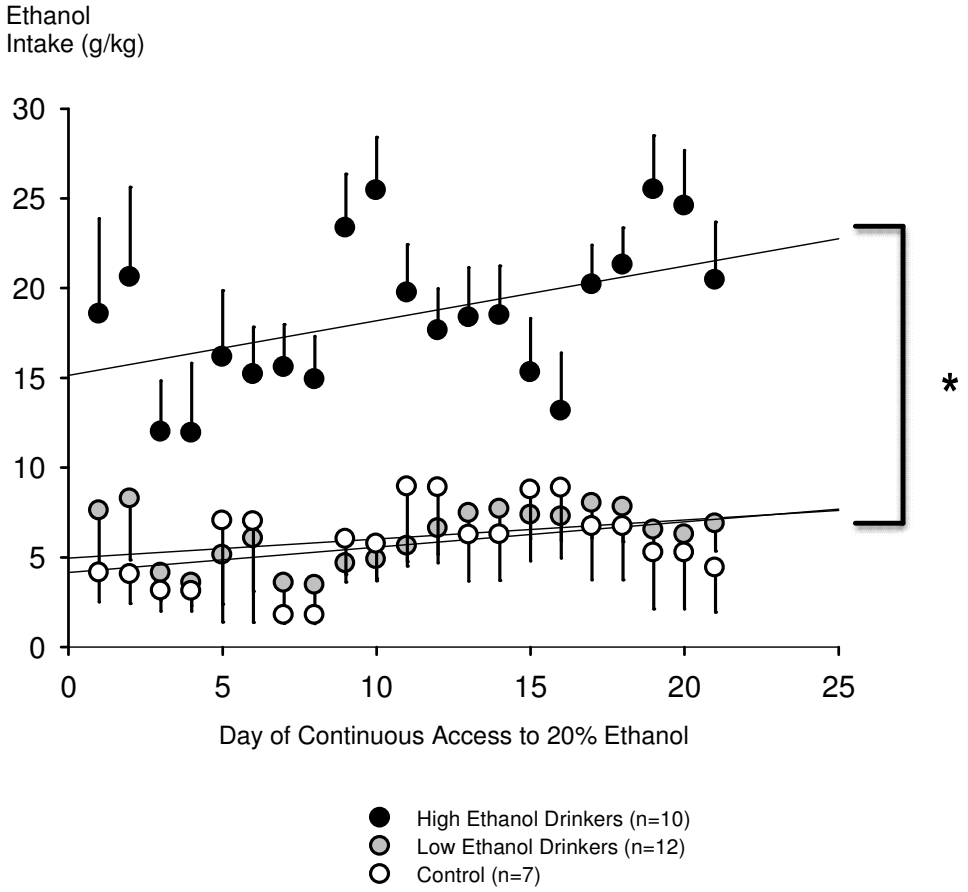
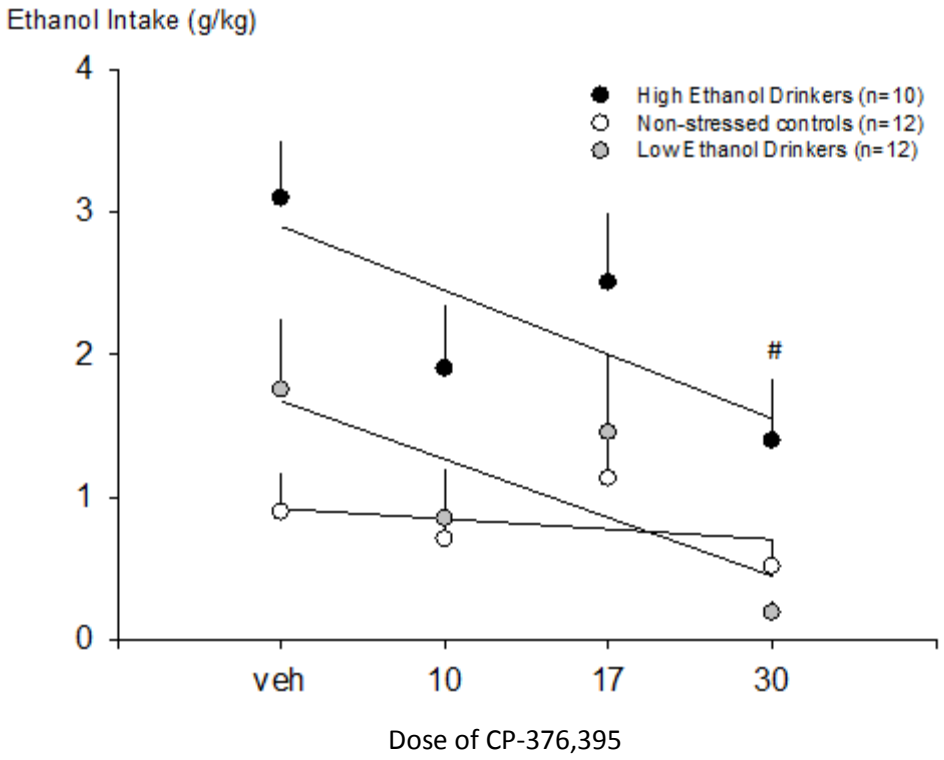
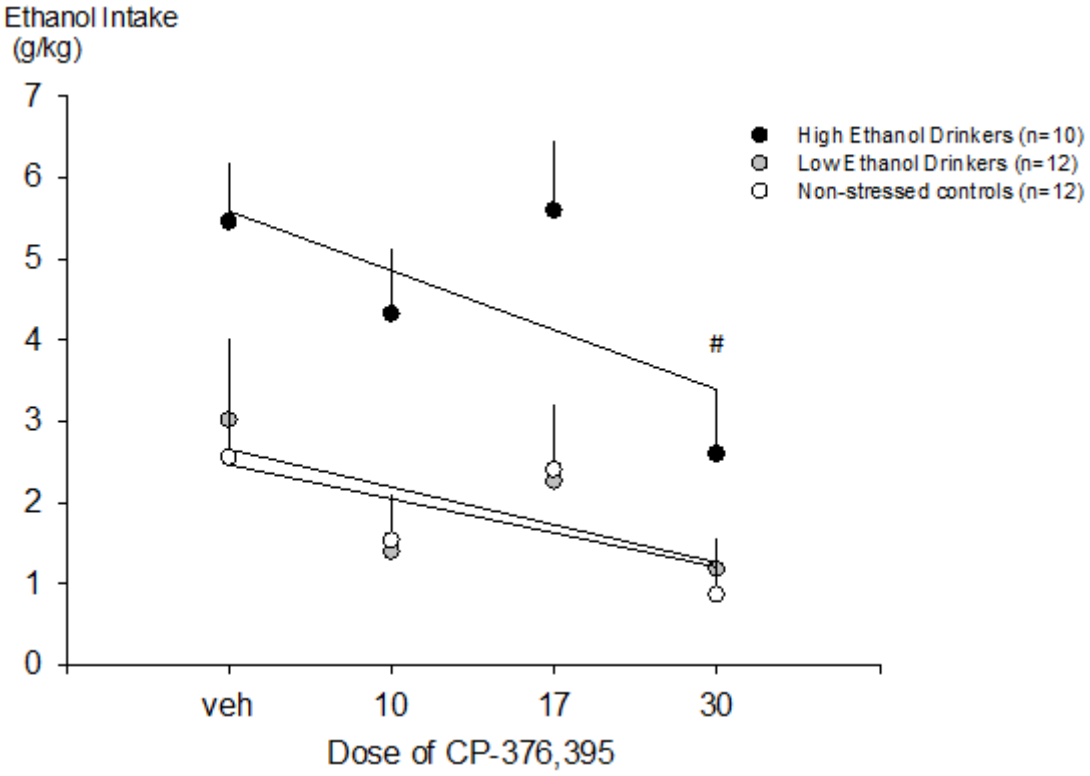


Figure 11. The effect of CP-376,395 (10, 17, 30mg/kg) on ethanol intake of high and low ethanol drinkers after differential lengths of ethanol access. Error bars indicate standard error of the mean. Pound sign (#) indicate significant decreases in ethanol drinking compared to vehicle (saline) A) 2 hour ethanol access. B) 4 hour ethanol access. C) 24 hour ethanol access.

A)



B)


Ethanol Intake
(g/kg)
7
6
5
4
3
2
1
0
High Ethanol Drinkers (n=10)
Low Ethanol Drinkers (n=12)
Non-stressed controls (n=12)
#
veh
10
17
30
Dose of CP-376,395

C)

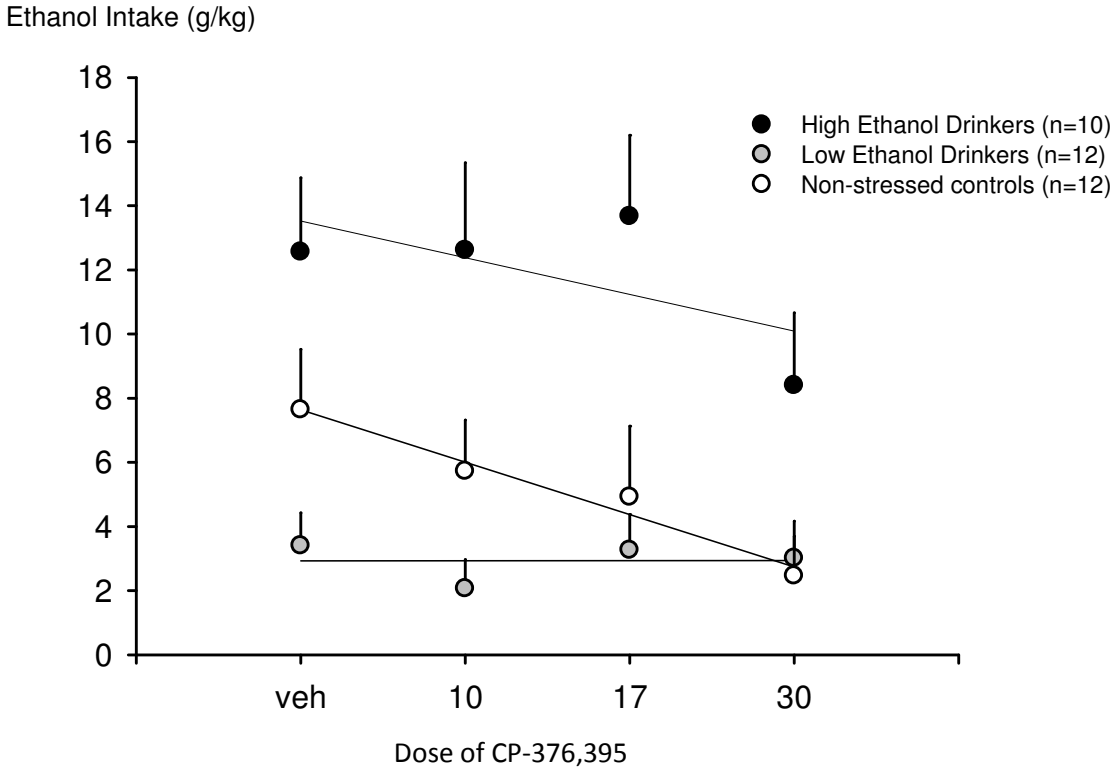
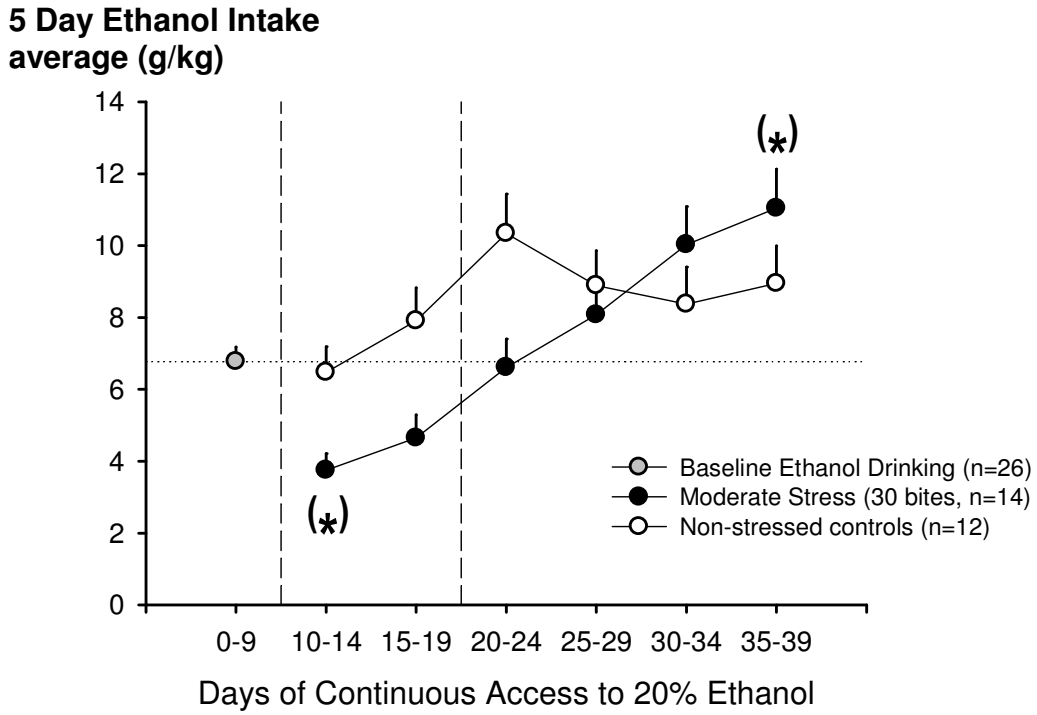

Ethanol Intake (g/kg)
18
16
14
12
10
8
6
4
2
0
High Ethanol Drinkers (n=10)
Low Ethanol Drinkers (n=12)
Non-stressed controls (n=12)
veh
10
17
30
Dose of CP-376,395

Table 5. Effects of Differential Doses of CP-376,395 on Water Intake at 2, 4, and 24 hours.
on water intake at 2, 4, and 24 hours.

Group	Dose	Time Period	Water Intake (ml)
Group	**Drug Dose (mg/kg)**	**EtOH Access**	**Water Intake (ml)**
High EtOH Drinkers	Vehicle	2 hr	0.81±0.10
		4 hr	1.92±0.28
		24hr	5.34±0.61
	10	2 hr	0.98±0.25
		4 hr	1.60±0.38
		24hr	5.66±0.86
	17	2 hr	1.17±0.23
		4 hr	1.78±0.35
		24hr	6.44±1.02
	30	2 hr	0.72±0.17
		4 hr	1.20±0.24
		24hr	5.44±0.93
Low EtOH Drinkers	Vehicle	2 hr	1.15±0.18
		4 hr	2.13±0.28
		24hr	6.58±0.95
	10	2 hr	0.96±0.17
		4 hr	1.85±0.28
		24hr	6.38±0.65
	17	2 hr	0.76±0.12
		4 hr	1.27±0.17
		24hr	6.39±0.60
	30	2 hr	0.67±0.06
		4 hr	1.16±0.16
		24hr	4.84±0.69
Control	Vehicle	2 hr	1.08±0.19
		4 hr	1.44±0.37
		24hr	6.15±0.83
	10	2 hr	1.11±0.28
		4 hr	1.63±0.49
		24hr	6.33±0.60
	17	2 hr	0.84±0.17
		4 hr	1.41±0.30
		24hr	7.25±0.60
	30	2 hr	1.33±0.45
		4 hr	2.03±0.67
		24hr	7.94±1.13

Figure 12. The effects of social defeat stress on previously established patterns of A) ethanol intake and B) ethanol preference ratio are examined. Baseline ethanol intake and ethanol preference ratio include data from all mice. Error bars indicate standard error of the mean.

A)



B)

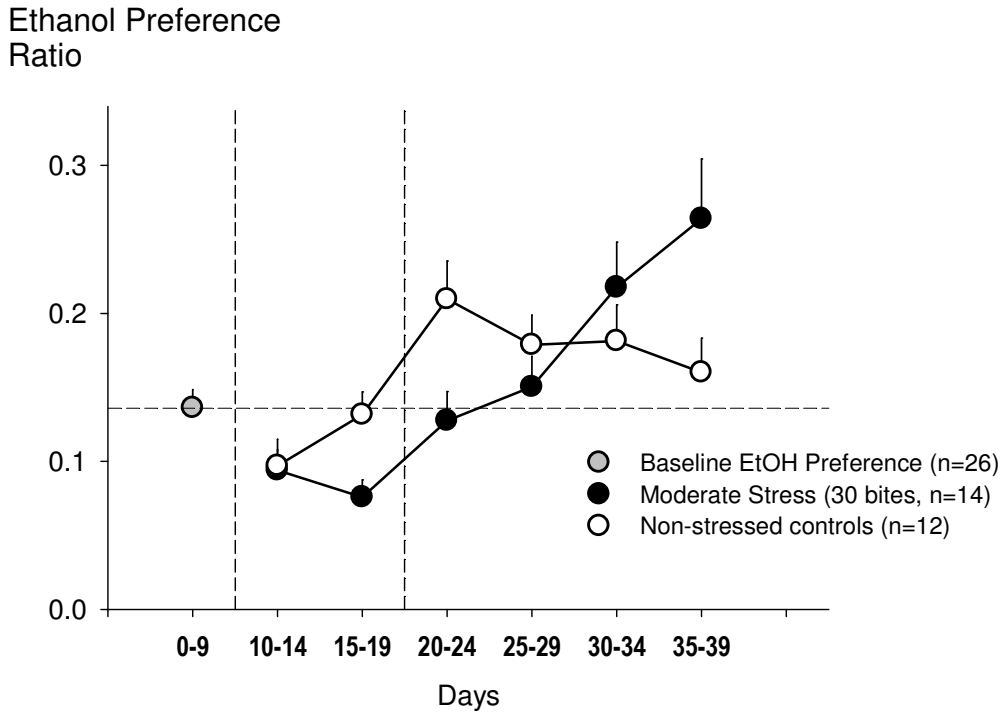

Ethanol Preference Ratio
0.3
0.2
0.1
0.0
0-9
10-14
15-19
20-24
25-29
30-34
35-39
Days
Baseline EtOH Preference (n=26)
Moderate Stress (30 bites, n=14)
Non-stressed controls (n=12)

Figure 13. The effects of the moderate social defeat stress on motivation for 20% ethanol during intermittent access to alcohol. CFW mice obtained reinforcements by a nose-poke in an operant conditioning procedure under a progressive ratio schedule.