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Understanding the role of the bed nucleus of the stria terminalis in alcohol use disorders

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UNDERSTANDING THE ROLE OF THE BED NUCLEUS OF THE STRIA TERMINALIS IN ALCOHOL USE DISORDERS

by

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DEDICATION

I would like to dedicate this to my wonderful husband, Jaeden Walker, for being patient and supportive through all the long nights.

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ABSTRACT

Alcohol Use Disorders (AUDs) have devastating economic, mortality, and public health implications on society. Repeated cycles of alcohol intoxication and abstinence are known to induce neuroplastic alterations in specific brain regions, alterations which in turn trigger and sustain excessive alcohol drinking. The Bed Nucleus of the Stria Terminalis (BNST) has been proposed as a critical brain site for neuroadaptations induced by chronic alcohol. The Pituitary Adenylate-Cyclase Activating Polypeptide (PACAP) system highly expressed in the BNST, has been proposed to be a master regulator of the stress response. These experiments aimed to investigate the role of the PACAP system of the BNST in alcohol drinking. Using a two-bottle choice chronic intermittent ethanol paradigm, we demonstrated that excessive intermittent alcohol consumption causes a marked increase in PACAP immunoreactivity in the BNST of mice. In addition, we observed a significant higher PACAP expression in the BNST of female, compared to male mice. These data lay the foundation for more extensive studies which may lead to the identification of a neuropeptide system with a critical role in heavy alcohol drinking. A deeper understanding of the specific neuroadaptations produced by

chronic alcohol will be essential for the discovery of novel therapeutic agents to alleviate alcoholism.

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ABBREVIATIONS

ACTH	Adrenocorticotropic Hormone
AUD	Alcohol Use Disorder
AVP	Arginine Vasopressin Pathway
BAL	Blood Alcohol Level
BNST	Bed Nucleus of the Stria Terminalis
CeA	Central Amygdala
CSF	Cerebrospinal Fluid
DAB	3,3'-diaminobenzidine
EPM	Elevated Plus Maze
FDA	Food and Drug Administration
GABA	Gamma-aminobutyric acid
MRI	Magnetic Resonance Image
NAcc	Nucleus Accumbens
NMDA	N-methyl-D-aspartate
PACAP	Pituitary Adenylate Cyclase-Activating Polypeptide
PVN	Paraventricular nucleus of the hypothalamus
VTA	Ventral Tegmental Area

INTRODUCTION

Alcohol Use Disorders

Alcohol Use Disorders, or AUDs, have devastating economic and public health implications on society. The World Health Organization's 2014 Global Status report on Alcohol and Health reports that 3.3 million deaths in 2012 were attributable to alcohol consumption. In addition to societal costs due to alcohol consumption, individuals also experience personal costs to themselves and their families. These costs are measured by the loss of life quality, pain and anguish, both emotional and physical (World Health Organization. Management of Substance Abuse Team. 2011). It is also important to note that AUDs occur more frequently in adult men (12.4%) than adult women (4.5%) however, females are more likely to reach higher blood alcohol levels (BALs) than their male counterparts consuming the same drink, because of both lower body weights and body water content, and higher body fat composition (American Psychiatric Association, 2013a).

AUDs are diagnosed based on the DSM-V, as meeting at least two of the eleven criteria listed below, within the last twelve months:

1) Alcohol is often taken in larger amounts or over a longer period than was intended; 2) There is a persistent desire or unsuccessful efforts to cut down or control alcohol use; 3) A great deal of time is spent in activities necessary to obtain alcohol, use alcohol, or recover from its effects; 4) Craving, or a strong desire or urge to use alcohol; 5) Recurrent alcohol use resulting in a failure to fulfill major role obligations at work, school, or home; 6) Continued alcohol use despite having persistent or recurrent social or

interpersonal problems caused or exacerbated by the effects of alcohol; 7) Important social, occupational, or recreational activities are given up or reduced because of alcohol use; 8) Recurrent alcohol use in situations in which it is physically hazardous; 9) Alcohol use is continued despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by alcohol; 10) Tolerance, as defined by either of the following: A need for markedly increased amounts of alcohol to achieve intoxication or desired effect, or a markedly diminished effect with continued use of the same amount of alcohol; and 11) Withdrawal, as manifested by either of the following: the characteristic withdrawal syndrome for alcohol or alcohol (or a closely related substance, such as a benzodiazepine) is taken to relieve or avoid withdrawal symptoms (American Psychiatric Association 2013b).

AUDs are highly comorbid with other psychiatric disorders such as schizophrenia, bipolar disorder, antisocial personality disorder, as well as depressive and anxiety disorders (American Psychiatric Association 2013a). Conversely, these psychiatric disorders may put individuals at risk for alcohol dependence when self-medication with alcohol is used to alleviate pre-existing symptoms (Gilpin and Koob 2008). Despite the high prevalence of AUDs, there remains a lack of diverse and effective treatments for alcohol dependence, or alcoholism.

Currently, there are only three FDA-approved drugs to treat AUDs: naltrexone, acamprosate, and disulfiram. Naltrexone is an opioid receptor blocker, which inhibits cravings and blocks the rewarding effects of alcohol (Liu et al. 2014). Acamprosate, the calcium salt of N-acetyl-homotaurin, is a synthetic analogue of the amino acid gamma-

aminobutyric acid (GABA) with a complex and still debated mechanism of action (Heilig 2014). Disulfiram is an aldehyde dehydrogenase inhibitor, which causes aversive reactions if alcohol is consumed due to acetaldehyde accumulation (Hald and Jacobsen 1948; Johansson 1992). However, not all patients respond to these drugs, and issues of side effects also reduce compliance.

Neurobiology of Alcohol Addiction

Addiction is a chronic, relapsing brain disease that is characterized by the compulsion to seek and take drug/alcohol, an inability to control the amount used, and a negative emotional state that arises when the substance cannot be accessed (Gilpin and Koob 2008). Alcohol addiction has been hypothesized to evolve from impulsive use to compulsive need through repeated cycles of intoxication and withdrawal, which result in profound neuroadaptations in specific areas of the brain (Cui et al. 2013; Koob 2013).

There are three widely recognized stages of alcohol use that overlap and ultimately contribute to the progressive transition from alcohol use to alcohol dependence. They are: 1) binge and intoxication, 2) withdrawal and negative affect, and 3) pre-occupation and anticipation, better known as craving (Volkow et al. 2016). Neuroadaptations within the addiction circuitry and the progressive transition from alcohol use to dependence hinge on the repeated cycling of these three stages.

Acute alcohol consumption stimulates the release of dopamine, which activates the brain reward circuitry. All addictive substances elicit this initial response (Di Chiara 2002; Koob 1992; Wise 2008). Research on the neurobiology of addiction has focused on

the positive reinforcing effects and the activation of the mesocorticolimbic dopamine system following acute drug administration. The mesocorticolimbic pathway is a dopaminergic pathway that connects the ventral tegmental area (VTA) to the nucleus accumbens (NAcc), as well as other limbic and cortical regions (Gilpin and Koob 2008). This circuit helps shape incentive salience, better known as the "wanting" or "desire" for alcohol (Berridge 2007; Robinson and Berridge 1993). In animal models of alcohol consumption, oral ethanol self-administration is reduced by the administration of dopamine and N-methyl-D-aspartate (NMDA) receptor antagonists into the NAcc (Rassnick et al. 1992). Alcohol ingestion as well as the anticipation of alcohol has been shown to cause an increase in extracellular dopamine levels in the NAcc (Weiss et al. 1993). However, it was also shown that 6-hydroxydopamine (6-OHDA)-induced lesions of the mesolimbic dopamine system is not sufficient to block alcohol self-administration (Rassnick et al. 1993). On the contrary, subjects undergoing alcohol withdrawal have been shown to display lower levels of dopamine, as compared to controls, suggesting a hypodopaminergic state (Karkhanis et al. 2015; Volkow et al. 2007).

In later stages, a shift from occasional alcohol use to alcohol dependence occurs, which parallels a switch from positive reinforcement to negative reinforcement as the motivating factor behind alcohol use; this switch is thought to be produced by neuroplastic changes elicited by chronic alcohol exposure (Gilpin and Koob 2008). Repeated exposure to the stress response leads to development of negative emotions and increased reactivity to stress (Davis et al. 2010; Jennings et al. 2013). In the withdrawal and negative affect stage, rewards are no longer the potent positive reinforcers they were

at the origin of drug use. The brain reward system becomes desensitized to drug and non-drug related rewards (Hagele et al. 2015; Hyatt et al. 2012; Konova et al. 2012) (Volkow et al. 2014; Volkow et al. 2006; Zhang 2013). This stage involves neurocircuitries of the extended amygdala and the activation of brain's stress neurotransmitter systems, such as the corticotropin-releasing factor (CRF) and the dynorphin (Volkow et al. 2016).

The last stage of addiction is the preoccupation and anticipation. This stage is characterized by profound deficits in prefrontal cortex function, which result in the serious impairment of judgment and self-regulation (Goldstein and Volkow 2011). In addition, the prefrontal cortex becomes hyperreactive to cues previously associated with alcohol.

Bed Nucleus of the Stria Terminalis (BNST) and its role in the actions of alcohol

The Bed Nucleus of the Stria Terminalis (BNST) is a brain structure located in the basal forebrain (Avery et al. 2016; Lebow and Chen 2016). It contains 12-18 subnuclei, each with diverse receptors, transporters, neurotransmitters, and proteins (Lebow and Chen 2016). BNST neurons have been shown to express different neuropeptides, such as CRF, enkephalin (ENK), neuropeptide Y (NPY), neurotensin, and somatostatin (SST). These neuropeptides show different immunoreactivity in each of the BNST subregions, confirming the diverse makeup and function of each subregion of the BNST (Walter et al. 1991). The BNST contains mostly GABAergic neurons (Kash 2012; Kozicz et al. 1997) and some glutamatergic neurons principally located in the ventral BNST (Daniel and Rainnie 2016; Jennings et al. 2013). The BNST acts as a key relay for many neuronal

pathways. It receives glutamatergic projections from thalamic, cortical, and amygdalar regions and GABAergic projections from the amygdalar region. The brainstem and hypothalamus send modulatory inputs to it, and then projects back to all these areas in varying degrees (Kash 2012).

It is also important to note that steroid hormones receptors such as androgen, estrogen (both alpha and beta subtypes) and progesterone are located in the BNST (Frazier et al. 2006; He et al. 2014; Laflamme et al. 1998), possibly influencing states of sustained fear and anxiety both sexes and attachment of proper valence to situations (Lebow et al. 2012). Steroid hormone manipulation affects anxiety-related behaviors; more specifically, progesterone reduces CRF-enhanced startle and stimulates BNST neurons via oxytocin (Wakerley et al. 1998). Male rats had reduced light enhanced startle because of testosterone. Because of these effects of testosterone, intact males hold the lowest light-potentiated startle; pregnant females possess the highest (Toufexis 2007). Anatomical sex differences also exist, such as human females boasting a 76% greater structural connectivity in the BNST (Avery et al. 2014). Male rodents possess a larger Arginine Vasopressin Pathway (AVP) from the BNST to the lateral septum (De Vries and al-Shamma 1990; Miller et al. 1989) and hypogonadism reduces BNST AVP immunoreactivity (Rosie et al. 1993; Viau et al. 2001). Sex differences in anatomy, anxiety-related receptors, and behavior of the BNST may contribute to the immense difference in human psychiatric diseases prevalence rates between men and women.

The BNST has been identified as a key component of the extended amygdala, which also includes the central nucleus of the amygdala (CeA) (Avery et al. 2016). These

brain regions have comparable neural inputs and outputs, neurochemical components, and cell types (Alheid et al. 1998). Though the BNST and CeA are similar, the BNST mediates sustained anxiety states and contextual fears, as opposed to the CeA, which plays a role in acute fear responses (Kash 2012; Waraczynski 2016). The CeA is responsible for responding to perceived immediate danger, or conditioned fear, which is signaled by discrete sensory cues, such as tones or light cues; BNST lesions do not effect this response in subjects, suggesting that conditioned fear responses are specific to the CeA (Gewirtz et al. 2000; LeDoux et al. 1988; Sullivan et al. 2004; Walker and Davis 1997).

Anterolateral BNST activation increases plasma levels of CRF (Dunn 1987); lesions on this area have been shown to lead to a decrease in CRF plasma levels (Herman et al. 1994) and subsequently to decreases in ACTH and corticosterone levels (Gray et al. 1993). Posterior and medial regions demonstrate the ability to cause anxiolytic effects (anxiolytic) (Dunn 1987; Herman et al. 1994) which validates the theory that different BNST subregions mediate different aspects of the stress responses.

Kim et al demonstrated that if GABAergic signals from the oval BNST to the anterodorsal BNST (adBNST) are active and glutamatergic signals from the basolateral amygdala (BLA) to the adBNST are inactive, glutamatergic projections from the adBNST to the parabrachial nucleus (PBN), ventral tegmental area (VTA), and the lateral hypothalamus (LH) are inhibited. This inhibition causes an elevated respiration, avoidance of stress-associated places, and a decrease in open field exploration. On the other hand, when the BLA glutamatergic projections activate, they activate the adBNST,

which projects GABAergic neurons to the PVN. The results are a decrease in respiration and an increase in open field exploration and place preferences (Kim et al. 2013).

This was also exemplified in a study carried out by Jennings et al by mapping the stimulated projections from the BNST to the non-dopaminergic neurons of the VTA, using channelrhodopsin-2 (CR-2). By photostimulating glutamatergic projections in the BNST, anxiogenic and aversive effects can be initiated. On the other hand, photostimulation of GABAergic projections produced anxiolytic and rewarding effects. Direct inhibition of GABA neurons in the VTA reconfirmed this point (Jennings et al. 2013).

Just as the BNST modulates chronic stress, chronic stress shapes BNST function through structural and functional changes. Expression of CRF in the BNST increases after chronic social and "mild" stress, in addition to chronic corticosterone treatment (Makino et al. 1994; Schulkin et al. 1994; Watts and Sanchez-Watts 1995). These stress-inducing paradigms have been implicated in BNST volume and dendritic length increases (Pego et al. 2008), as well as account for a greater number of BNST dendritic branches (Vyas et al. 2003; Vyas et al. 2002). These stress response reactionary neuroadaptations could be responsible for the development of anxiety disorders, such as post-traumatic stress disorder (PTSD), general anxiety disorder (GAD), or antisocial behavior and aggression (Lebow and Chen 2016), as well as addiction disorders(Avery et al. 2016).

Because of the high comorbidity of alcohol use disorders and anxiety disorders—75% of people that have an AUD reported having a current or prior anxiety disorder diagnosis (Kushner et al. 2000; Menary et al. 2011; Swendsen et al. 2010)—the BNST

has become a brain region of interest as a possible pharmacological target to alleviate alcohol dependence as it is a pivotal structure involved in anxiety states. Several studies assessing acute ethanol intake have posited ethanol intake derives from its anxiolytic effect (Kushner et al. 2000; Wilson et al. 2004). Subjects with elevated anxiety states are significantly more like to engage in increased alcohol drinking in two-bottle choice paradigms (Primeaux et al. 2006; Spanagel et al. 1995); chronic anxiety may inspire alcohol abuse and lead to the transition to alcohol dependence (Koob 2003). Additionally, studies have shown that Alcohol-preferring (P) rats and Sardinian alcohol-preferring (sP) rats exhibit enhanced anxiety-like behavior on the EPM, as opposed to non-preferring (NP and sNP) lines (Colombo et al. 1995; Stewart et al. 1993). Recurring cycles of ethanol exposure and withdrawal have been linked to enhanced anxiety states (Kliethermes 2005; Valdez et al. 2002). Alcohol use to alleviate anxiety starts a vicious feed-forward cycle that leads to an allostatic state that increases both alcohol use and anxiety symptoms (Koob 2008).

The BNST has been associated with the negative reinforcement aspect of alcohol withdrawal. CRF in the CeA and BNST increases during ethanol withdrawal in dependent rats through hyperactive non-hypothalamic CRF systems (Funk et al. 2006; Merlo Pich et al. 1995; Olive et al. 2002; Zorrilla and Koob 2004). One study used central injections to administer CRF-receptor antagonists into ethanol dependent animals demonstrated a reduction of withdrawal-induced anxiety-like behavior and ethanol self-administration (Valdez et al. 2002), suggesting a relationship between ethanol withdrawal and the body's stress system. When administered directly into the BNST, CRF receptor

antagonists also attenuate anxiety-like behavior (Rassnick et al. 1993), as well as ethanol self-administration (Funk et al. 2006).

The BNST also plays a role in the reinstatement of substance use after periods of abstinence (Koob and Le Moal 2005). Elevations of the early gene c-Fos immunoreactivity (marker of neuronal activation) were observed in the BNST following the presentation of drug-associated cues (Hill et al. 2007; Mahler and Aston-Jones 2012).

Pituitary Adenylate Cyclase-Activating Peptide (PACAP)

PACAP was discovered in ovine hypothalamus tissues because of its ability to increase adenylyl cyclase activity in the anterior pituitary (Hammack and May 2015; Miyata et al. 1989). It is a member of the glucagon/vasoactive intestinal peptide (VIP)/secretin peptide superfamily. The PACAP gene's structure is similar to that of the VIP gene (68%) (Lamperti et al. 1991; Miyata et al. 1989), suggesting the genes share a common ancestry. PACAP has been shown to have 1000 times the adenylyl cyclase activating power as VIP (Miyata et al. 1990). PACAP expression can be found in primitive chordates, meaning that its structure and function has been evolutionarily preserved (Hammack and May 2015; Sherwood et al. 2000; Vaudry et al. 2009).

There are two variations of the PACAP peptide, PACAP-27 (27 amino acids) and PACAP-38 (38 amino acids), but PACAP-38 is more profuse throughout the body, including the central nervous system (Arimura et al. 1991; Hammack and May 2015; Miyata et al. 1990). Out of the total peptide found in brain tissue, PACAP 27 represents only 10% (Arimura et al. 1991; Ghatei et al. 1993; Masuo et al. 1993; Mikkelsen et al.

1995; Piggins et al. 1996).There are currently two classifications for PACAP receptors identified: Type I binding sites, which were identified using 125I-PACAP27 as a radioligand in the anterior pituitary and hypothalamus exhibit and have a high affinity for PACAP38 and PACAP27 (*K*d ' 0.5 nM), but a low affinity for VIP (*K*d .<500 nM) (Cauvin et al. 1990; Gottschall et al. 1991; Gottschall et al. 1990; Lam et al. 1990; Suda et al. 1992). Type II binding sites, which have a similar affinity for PACAP and VIP (*K*d ' 1 nM) and found in the brain, as well as peripheral organs, such as the lung, duodenum, and thymus (Gottschall et al. 1990; Hammack and May 2015; Lam et al. 1990; Vaudry et al. 2000).

The densest area of PACAP binding sites and PACAP-containing neurons is found in the parvo- and magnocellular neurons of paraventricular and supraoptic nuclei of the hypothalamus, which reveals PACAP's role in neuroendocrine function (Ando et al. 1994; Hammack et al. 2010; Kimura et al. 1994; Kivipelto et al. 1992; Ko¨ves K 1991; Mikkelsen et al. 1995; Piggins et al. 1996; Vaudry et al. 2000). PACAP-immunoreactive fibers are densely present in the internal zone of the median eminence and the area of the capillaries of the hypothalamohypophysial portal system (Hannibal et al. 1995; Kivipelto et al. 1992; Ko¨ves K 1991; Mikkelsen et al. 1995). Radioimmunoassay (RIA) quantification has revealed that in the rat portal blood PACAP concentrations are significantly higher than in peripheral blood; this shows PACAP is transported to the pituitary when excreted by hypothalamic nerve terminals (Dow et al. 1994). PACAP containing neurons are not limited to the hypothalamus; they also reside in the substantia nigra, nucleus accumbens, septum, globus pallidus, cerebral piriform cortex, pons,

hippocampus, olfactory nuclei, and distinct areas of the amygdala and BNST (Ghatei et al. 1993; Hammack et al. 2010; Masuo et al. 1993; Vaudry et al. 2000).

Many studies have concluded that the behavioral response to stress is heavily arbitrated by the PACAP system. Areas of the brain that respond to stress and anxiety are highly populated with PACAP and its receptor PAC1 (Arimura et al. 1991; Lezak et al. 2014a; Nomura et al. 1996; Shioda et al. 1997). Intracerebroventricularly (i.c.v.)administration of PACAP, in addition to direct administration into the CeA, PVN, and BNST, stimulates a stress response and the hypothalamic–pituitary–adrenal (HPA) axis, as well as extrahypothalamic corticotropin-releasing factor (CRF) systems (Agarwal et al. 2005; Dore et al. 2013; Missig et al. 2014; Norrholm et al. 2005).

PACAP fibers synapse on hypothalamic PVN CRF neurons (Legradi et al. 1998) and stimulate CRF production and excretion (Agarwal et al. 2005). One study showed that after restraint, PACAP null mice exhibited no upregulation of PVN CRF mRNA, as opposed to their control counterparts (Stroth and Eiden 2010). This means CRF production and release is stimulated by upstream signals from PACAP (Hammack and May 2015). PACAP uses the CRF system to exert its effects (Dore et al. 2013). This was demonstrated in mice lacking the PACAP and PAC1 receptor gene exhibited weakened HPA activation after chronic stress exposure (Hashimoto et al. 2009; Hattori et al. 2012; Stroth and Eiden 2010), decreased anxiety behavior (Girard et al. 2006; Hashimoto et al. 2001; Otto et al. 2001) and a decline in contextual fear conditioning (Lezak et al. 2014a; Otto et al. 2001). In addition, infusions of PACAP in the Intra-BNST caused anorexic behavior in rats (Kocho-Schellenberg et al. 2014) and anxiety-like behavior (Hammack et

al. 2009; Lezak et al. 2014a). In addition, research that observed the effects of repeated stress concluded that expression of PACAP and PAC1 receptors were increased in the BNST after undergoing repeated stressors, and these changes may underlie the behavioral consequences of stress exposure (Hammack et al. 2009; Hammack et al. 2010; Lezak et al. 2014b).

The goal of this study was to investigate the effects of intermittent, chronic alcohol on PACAP expression in the BNST in both males and female mice. Future experiments will elucidate the functional role of these neurochemical alterations in both excessive drinking as well as anxiety-like behavior in this animal model.

METHODS

Subjects

Subjects of this study were male and female C57BL/6J mice, 7 weeks upon arrival (Jackson Laboratory Bar Harbor, Maine). Subjects were housed in an AAALAC-approved vivarium on a 12-h light-dark cycle (lights off at 10:00 a.m.) with water and regular rodent chow available *ad libitum*. Experiments were conducted during the mice' dark cycle. Procedures adhered to the National Institutes of Health *Guide for the Care of Use of Laboratory Animals* and the *Principles of Laboratory Animal Care* and were approved by Boston University Medical Campus Institutional Animal Care and Use Committee.

Intermittent Access 20% Ethanol Two Bottle Choice (MWF)

Ethanol solutions (20% *v/v*) were prepared using 200 proof ethyl alcohol (Pharmco-Aaper) and tap water. Mice were individually housed, and then presented with two 50-ml Falcon tubes with rubber stoppers containing stainless steel 5 cm ball-bearing sipper tubes ("bottles"), both containing water. 3-5 days later, mice were given intermittent access to 20% (*v/v*) ethanol and water every Monday, Wednesday, and Friday, for 24 hours at the beginning of the dark cycle, for 10 consecutive weeks (Fig. 1). Bottles were presented to mice at the beginning of the dark cycle (10:00 am), and were weighed to the nearest hundredth of a gram both before presentation and 24 hours later, shortly before the lights go off. To control for spillage due to experimenter handling or evaporation, loss of fluid was assessed in empty cages. During the "off" days, bottles

were replaced by two water bottles. Mice were weighed to the nearest hundredth of a gram before every ethanol drinking session to calculate the grams of ethanol intake per kilogram of body weight.

Fig. 1

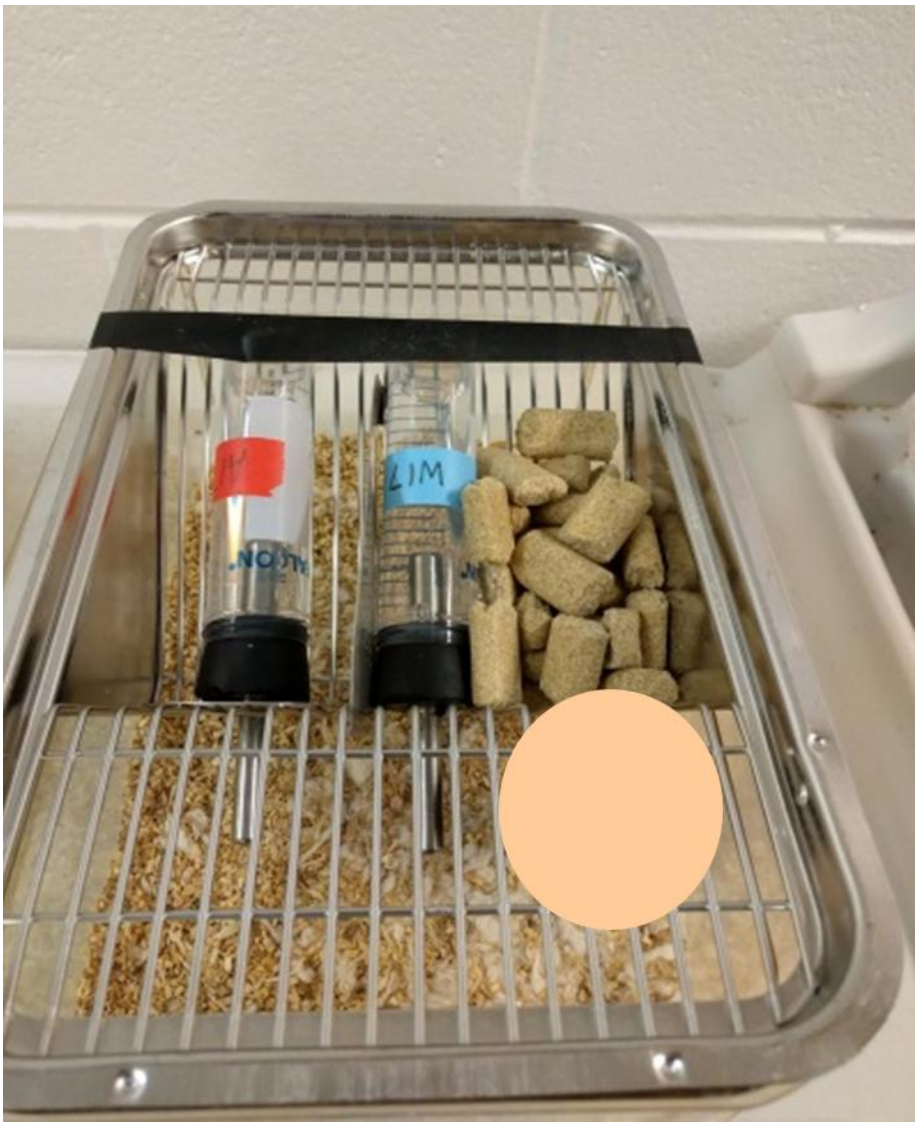


Fig. 1: Chronic, intermittent alcohol, two-bottle choice paradigm in the mouse home cage.

Defensive Withdrawal

To determine whether anxiogenic-like behavior could be observed during withdrawal from ethanol, mice underwent a defensive withdrawal task, 24-30 hours after the end of the previous ethanol access. The testing arena was a clear polyvinylchloride open field (106 cm × 92 cm × 77 cm) containing a cylindrical darkened "withdrawal" chamber (a 250-mL Pyrex beaker shielded from the outside light by wrapping in black tape). The "withdrawal" chamber was placed 15 cm from a corner facing the open arena, and testing occurred under room light (~200 lx). Mice were habituated to an anteroom adjacent to the testing room for at least 60 min. After habituation, mice were placed individually into the withdrawal chamber, facing the back, and then allowed to freely explore the arena for 10 min. After every trial, the maze was thoroughly cleaned with 70% ethanol solution and dried by paper towels. Sessions were video recorded for subsequent scoring of latency to emerge from the withdrawal chamber, number of withdrawals into the chamber, and total time spent in the chamber by treatment-blind raters.

Elevated Plus Maze

As a second test to assess anxiety-like behavior, the elevated-plus maze (EPM) test was used. The apparatus consisted of two open and two enclosed horizontal perpendicular arms (30 × 5 cm), positioned 40 cm above the floor. Open arms were dimly illuminated (1.5-2 lux). Mice were habituated to an anteroom adjacent to the testing room for at least 60 min. After habituation, mice were individually placed in the center of the

maze, facing one of the open arms, and allowed to explore for 5 min. After every trial, the maze was thoroughly cleaned with 70% ethanol solution and dried by paper towels. Sessions were video recorded for subsequent scoring of the number of entries into open arms, the time spent in open arms, the number of entries into closed arm, and the time spent in closed arms, by treatment-blind raters.

Perfusions

The mice were anesthetized with isoflurane and transcardially perfused with phosphate buffered saline first, followed by 4% paraformaldehyde (PFA). Brains were then collected, placed in PFA overnight, and then stored in a 30% sucrose solution in PBS at 4 °C until saturation.

Immunohistochemistry

Brains were cut into 30 μm coronal sections using a cryostat, and stored in a cryoprotectant solution at −20 °C until processed for immunohistochemistry. Every 4th section (120 μm apart; bregma +0.38 to +0.08 mm) of the BNST was collected in a systematic manner and processed for immunohistochemistry. After rinsing, incubation in 0.3% hydrogen peroxide TBS solution to quench endogenous peroxidases, additional rinsing, and a blocking step (3% normal goat serum, 0.4% Triton X-100), sections were incubated in an anti-PACAP primary antibody in blocking solution for 24 hr at 4 °C (1:1,000, Bachem, CA). Sections were then rinsed and incubated in a biotinylated anti-rabbit secondary antibody (1:500, Vector Labs, Burlingame, CA) in blocking solution,

for 2 hr at room temperature. Sections were washed and then incubated in an avidin–biotin horseradish peroxidase solution (Vector Labs, Burlingame, CA) and immunoreactivity was visualized using a diaminobenzidine (DAB) substrate kit (Vector Labs, Burlingame, CA) according to the manufacturer's instructions. Slides were dehydrated using graded alcohol concentrations and then cover-slipped.

PACAP Quantification

The relative density of PACAP-immunoreactivity quantification was performed using an Olympus (Center Valley, PA, USA) BX-51 microscope equipped with a Rotiga 2000R live video camera (QImaging, Surrey, BC, Canada), a three-axis MAC6000 XYZ motorized stage (Ludl Electronics, Hawthorne, NY, USA), and a personal computer workstation. Bright-field digital images of the anterior BNST were taken at 10× magnification using identical light intensity and exposure times. All images were imported into ImageJ software and two different area contours were drawn for each digital image. One tracing included the BNST positive PACAP immunoreactivity area and the other was taken for nonspecific DAB background with no specific PACAP staining. Mean optical density was obtained by subtracting the nonspecific DAB background from the positive PACAP immunoreactivity.

Statistical analyses

Ethanol drinking was analyzed using a three-way ANOVA (Session as a within-subject factor, Group and Sex as between-subjects factors). Data from anxiety tests and

from PACAP immunoreactivity were analyzed using a two-way ANOVA (Sex and Group as between subjects factors). Statistical significance was set at $p<0.05$. The software/graphic packages used were SigmaPlot 11.0 and Statistica 7.0.

RESULTS

Ethanol drinking in the intermittent access paradigm

Mice subject to chronic, intermittent access to a 20% v/v ethanol solution and water escalated their alcohol intake over time during the 4 weeks (12 sessions) under observation (Session: $F(17,306)= 5.56$, $p<0.001$). Female mice consumed significantly more ethanol than male mice and showed steeper escalation (Sex: $F(1,18)= 124.40$; $p<0.001$; Sex*Session: $F(17,306)= 2.58$; $p<0.001$; Fig 3). By the third day of ethanol access, females were consuming significantly more ethanol than males. Average ethanol intake across the 6 weeks was higher in female mice, compared to their male counterparts (21.4 vs. 13.1 g/kg, respectively). Water intake did not significantly differ between sexes (Sex: $F(1,18)= 0.05$; n.s.), and decreased over time (Session: $F(17,306)= 4.04$; $p<0.001$), although similarly in the two sexes (Sex*Session: $F(17,306)= 0.54$; n.s.; Fig 3). Average water intake across the 6 weeks was 57.9 and 59.2 ml/kg in females and males, respectively. Ethanol preference over time differed between sexes (Sex: $F(1,18)= 15.95$; $p<0.001$; Fig. 2), and increased over time (Session: $F(17,306) = 5.07$; $p<0.001$), although similarly in the two sexes (Sex*Session: $F(17,306)= 1.08$; n.s.). Average ethanol preference across the 6 weeks was significantly higher in females (70.2% vs. 58.9% in females and males, respectively). Finally, total fluid intake (ml/kg) decreased across days (Session: $F(17,306)= 2.97$, $p<0.001$). Fluid intake was higher in females (Sex: $F(1,18)= 80.09$; $p<0.001$; Sex*Session: $F(17,306)= 1.66$, $p<0.05$). Average fluid intake across days was 193.5 and 142.3 ml/kg in females and males, respectively.

Fig. 2

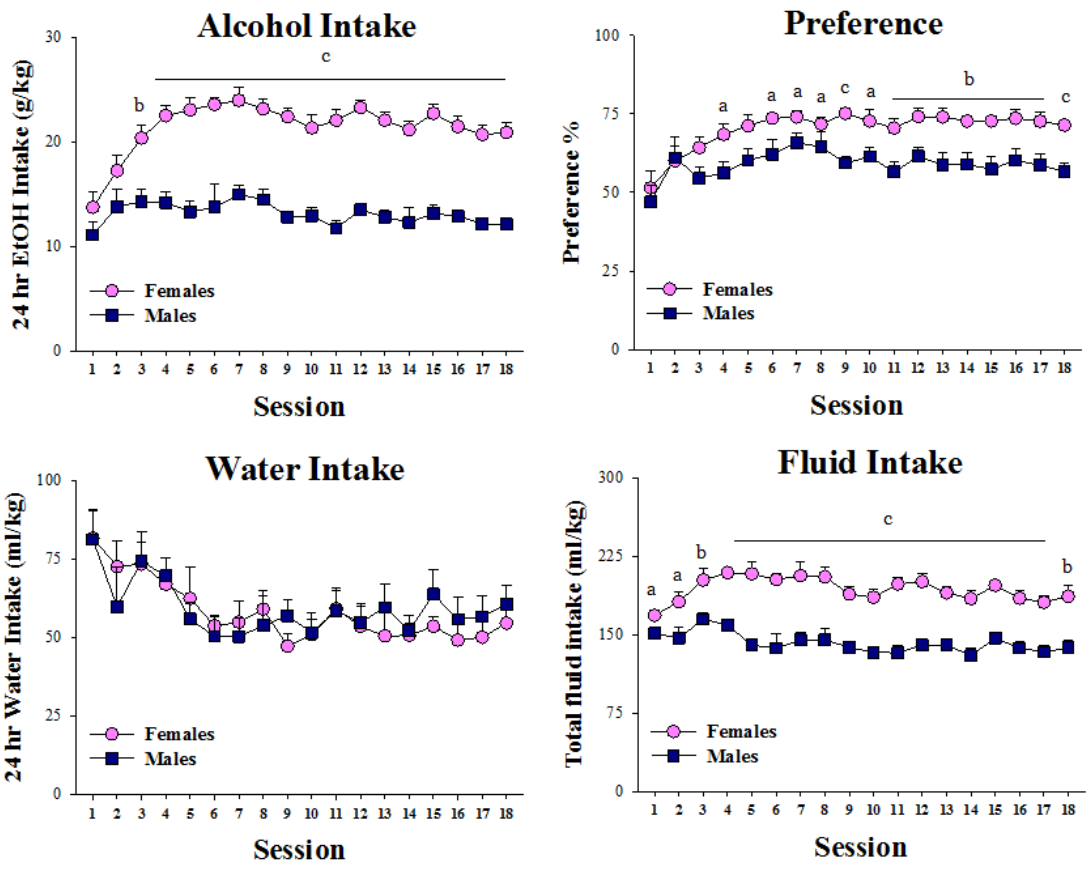


Fig. 2: Male and female C57Bl/6 mice were given access to intermittent access to ethanol for 18 sessions, i.e. 6 consecutive weeks (Monday, Wednesday, Friday, 24/hr a day, 20% *v/v* alcohol vs. water) (N = 10/group). A) Both female and male mice escalated ethanol intake; female showed higher ethanol intake than males. B) Water intake did not differ by sex and decreased over time. C) Female mice had a higher preference for ethanol than males; preference increased over time. D) Total fluid intake was higher in females. Data are Mean +SEM. Symbols a, b, and c denote p <0.05, p <0.01, p <0.001 vs. males, respectively.

Anxiety-like behavior in ethanol-withdrawn mice: Defensive withdrawal

As shown in Fig. 3, an effect of sex could be observed in the latency to first exit the withdrawal chamber (Sex: $F(1,35)= 9.42$, $p<0.01$); indeed, females displayed lower latency to exit compared to control males. However, ethanol had no effect on latency (Group: $F(1,35)= 0.11$, n.s.; Sex*Group: $F(1,35)= 0.07$, n.s.). There was also an effect of sex on the total time spent in the withdrawal chamber (Sex: $F(1,35)= 11.37$, $p=0.002$), where females spent less time inside the chamber compared to males (Fig. 4). Ethanol had no effect on this measure (Group: $F(1,35)= 1.06$, n.s.; Sex*Group: $F(1,35)= 0.39$, n.s.). Alcohol had an effect on the average time spent in the open field during each visit (Group: $F(1,35) = 8.78$, $p=0.005$; Sex*Group: $F(1,35)= 1.92$, n.s.), in that ethanol-drinking males spent longer time outside in each visit compared to controls (Fig. 5). Sex, however, had no effect on this measure (Sex: $F(1,35)= 0.98$, n.s.; Sex*Group: $F(1,35)= 1.92$, n.s.). Ethanol had an effect also on the number of transitions from chamber to open arena (Group: $F(1,35) = 12.21$, $p=0.001$), in that alcohol drinking males showed a reduction in number of transitions compared to controls (Fig. 6). Sex had no effect on this measure (Sex: $F(1,35)= 0.02$, n.s.; Sex*Group: $F(1,35)= 1.71$, n.s.).

Fig. 3

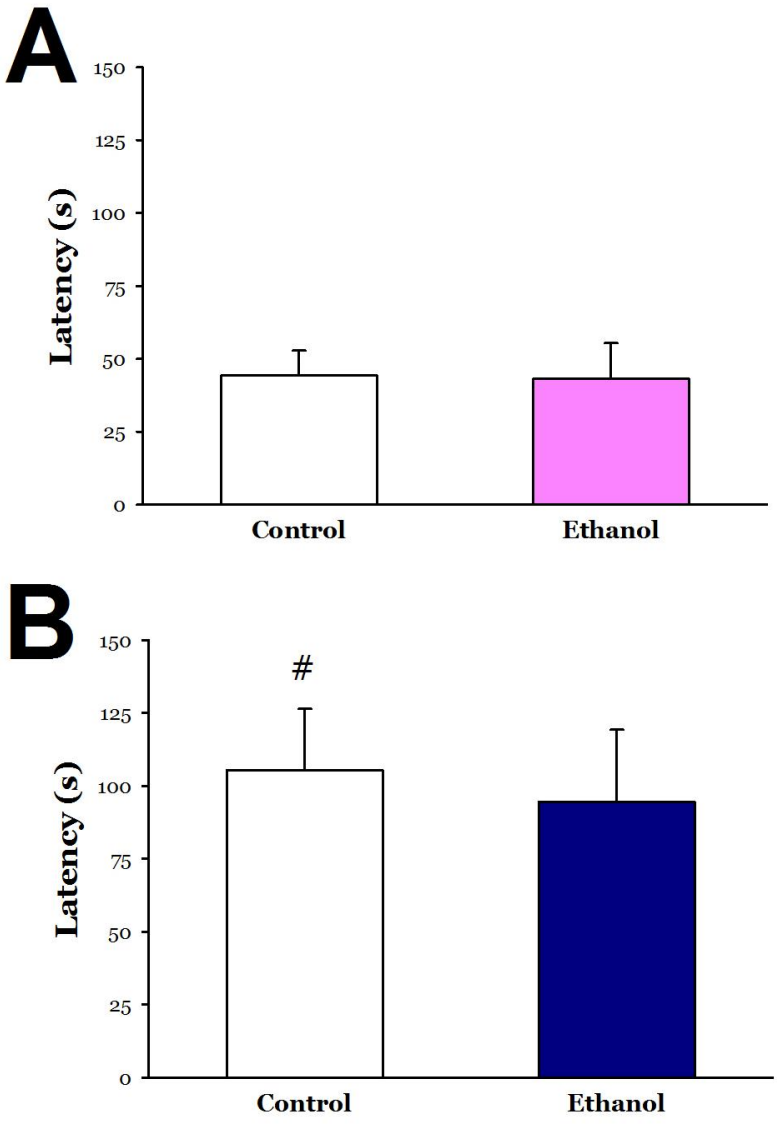


Fig. 3. Latency to first exit the chamber in the defensive withdrawal test A) Female control mice vs. alcohol-drinking mice showed no differences in latency B) Male control mice vs. alcohol-drinking mice had equivalent latencies; male controls had a higher latency than female control mice. Data are Mean +SEM. # p <0.05 vs. females of same treatment group.

Fig. 4

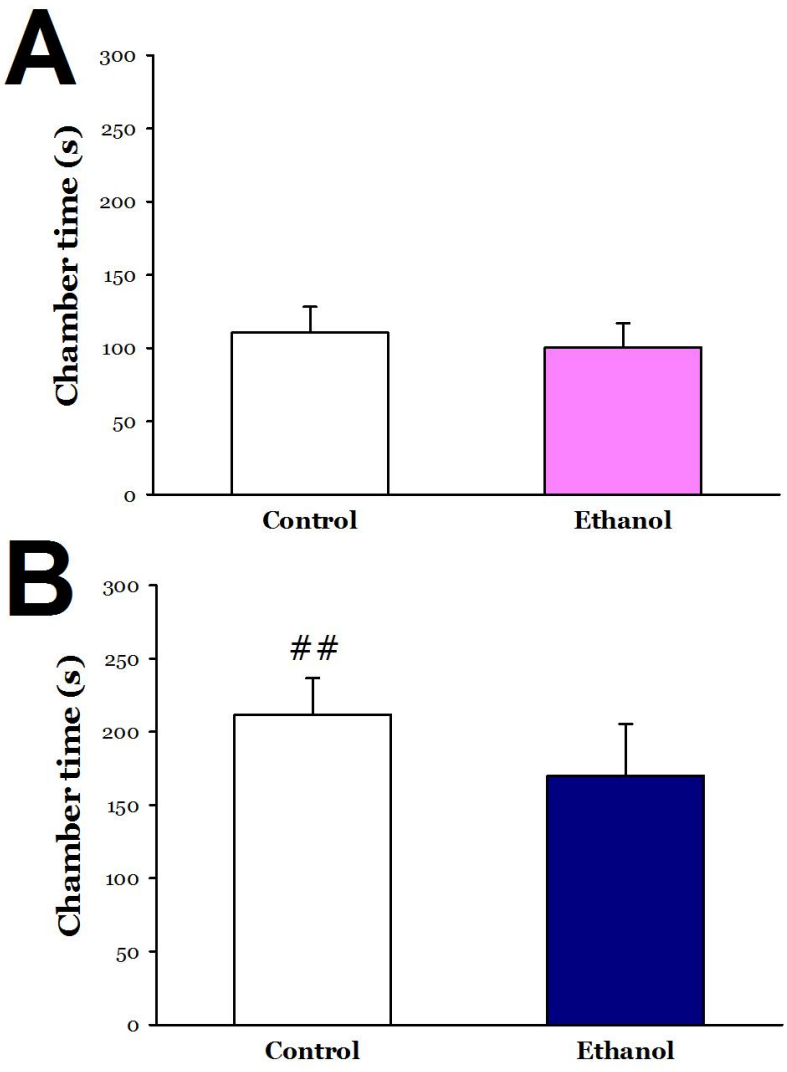


Fig. 4: Total time spent in chamber A) Female control mice vs. ethanol drinking females were not different in the total time spent in the chamber. B) Male control mice vs. ethanol drinking males were not different in the total time spent in the chamber. Data are Mean +SEM. ## p <0.01 vs. females of same treatment group.

Fig. 5

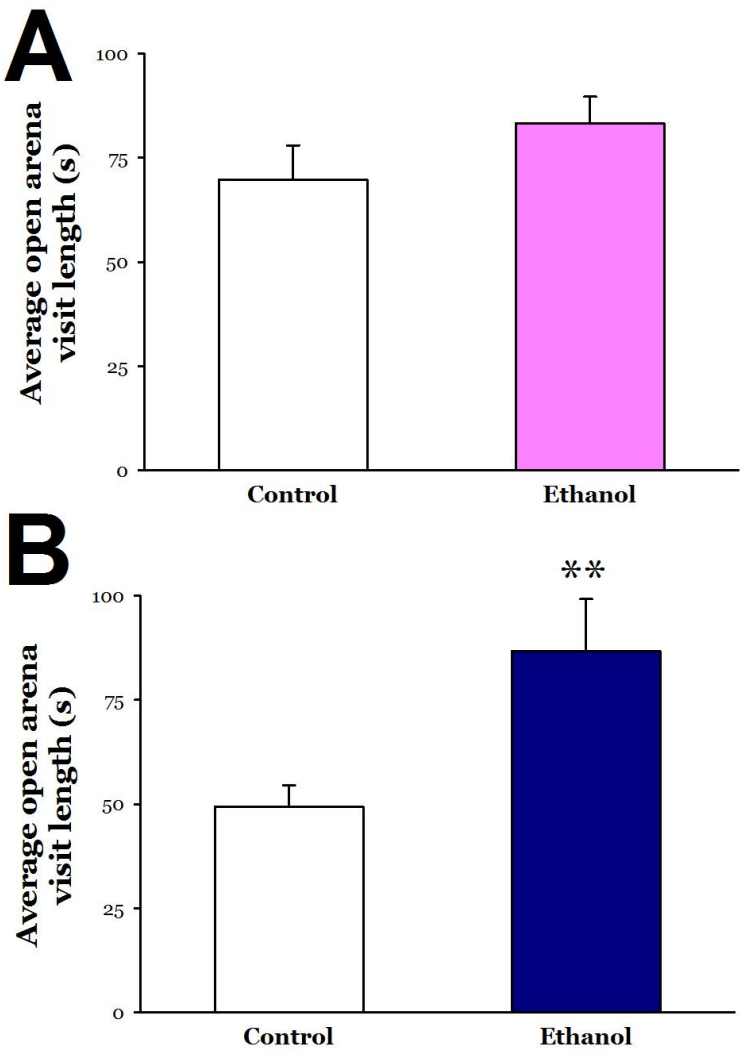


Fig. 5 Average open arena visit length A) Female control mice vs. ethanol drinking group had no difference in average arena visit length. B) Male ethanol drinking mice had significantly longer arena visits than the male controls. Data are Mean +SEM. ** p <0.01 vs. Control.

Fig. 6

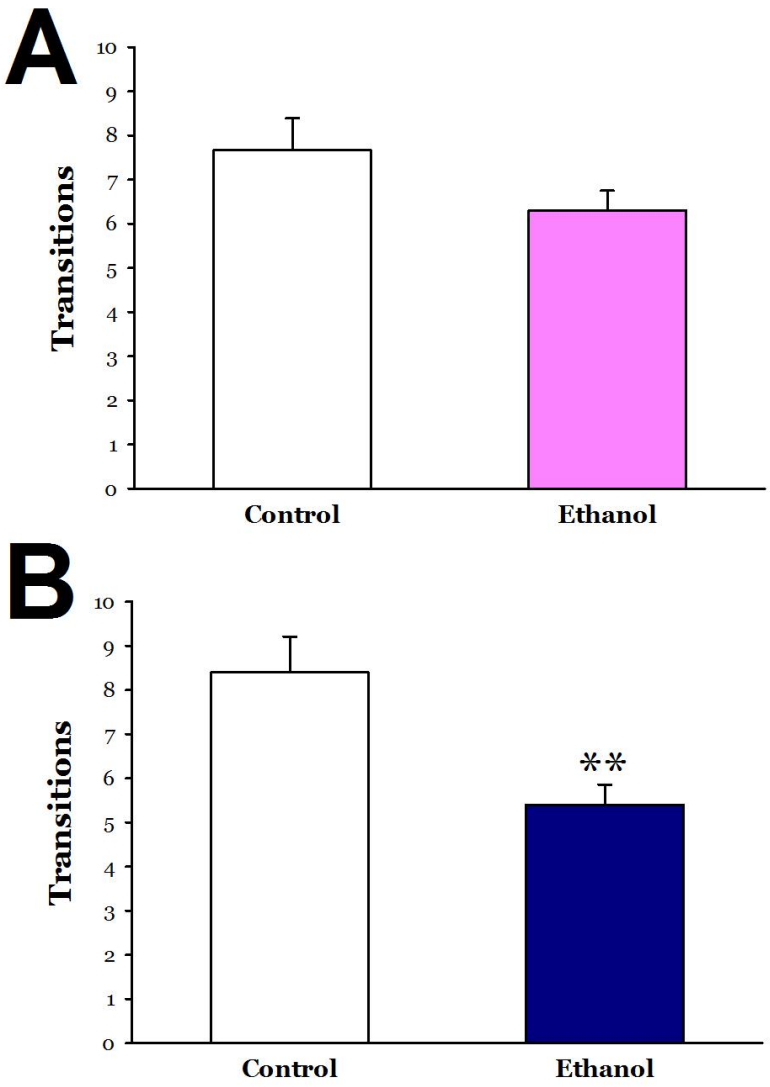


Fig. 6. Total transitions in and out of the withdrawal chamber A) Female control mice vs ethanol drinking group had similar number of transitions. B) Male control mice showed less transitions than the male ethanol drinking group. Data are Mean +SEM. ** *p* <0.01 vs. Control.

Anxiety-like behavior in ethanol-withdrawn mice: Elevated Plus Maze

An effect of sex*Group in percentage of the total arm time spent on the open arms (Sex*Group: $F(1,36)= 6.31$, $p<0.017$; Sex: $F(1,36)= 1.66$, n.s.; Group: $F(1,36)= 0.82$, n.s.) (Fig. 7). Post-hoc analysis showed that ethanol-drinking male mice spent more time in the open arms compared to controls. No effects were observed on closed arm entries (Sex: $F(1,36)= 0.00$, n.s.; Group: $F(1,36)= 2.30$, n.s.; Sex*Group: $F(1,36)= 0.02$, n.s.) (Fig. 8), indicating no differences in locomotor activity among groups.

Fig. 7

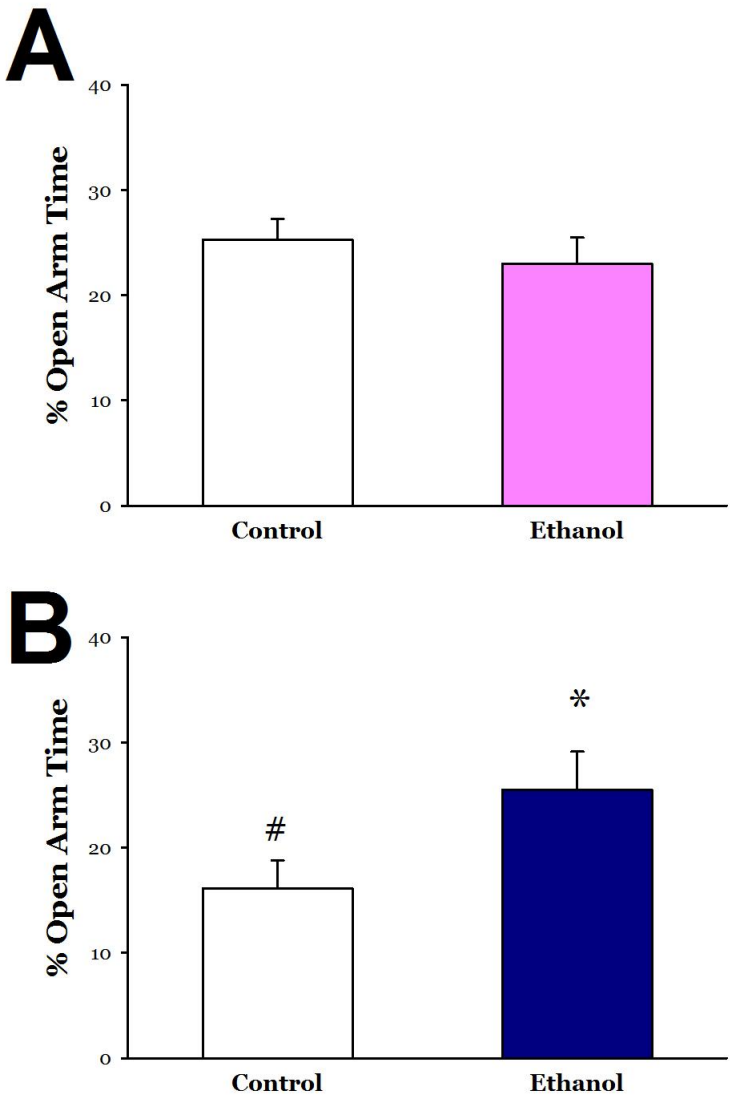


Fig. 7. Percent time in open arms. A) Female control mice vs. ethanol drinking group had similar open arm time. B) Control male mice spent less time on the open arms than female control mice, and ethanol-drinking male mice spent a higher percentage of time in the open arms compared to male control mice. Data are Mean +SEM. #p<0.05, vs. females of same treatment group, *p <0.05 vs. controls of same sex.

Fig. 8

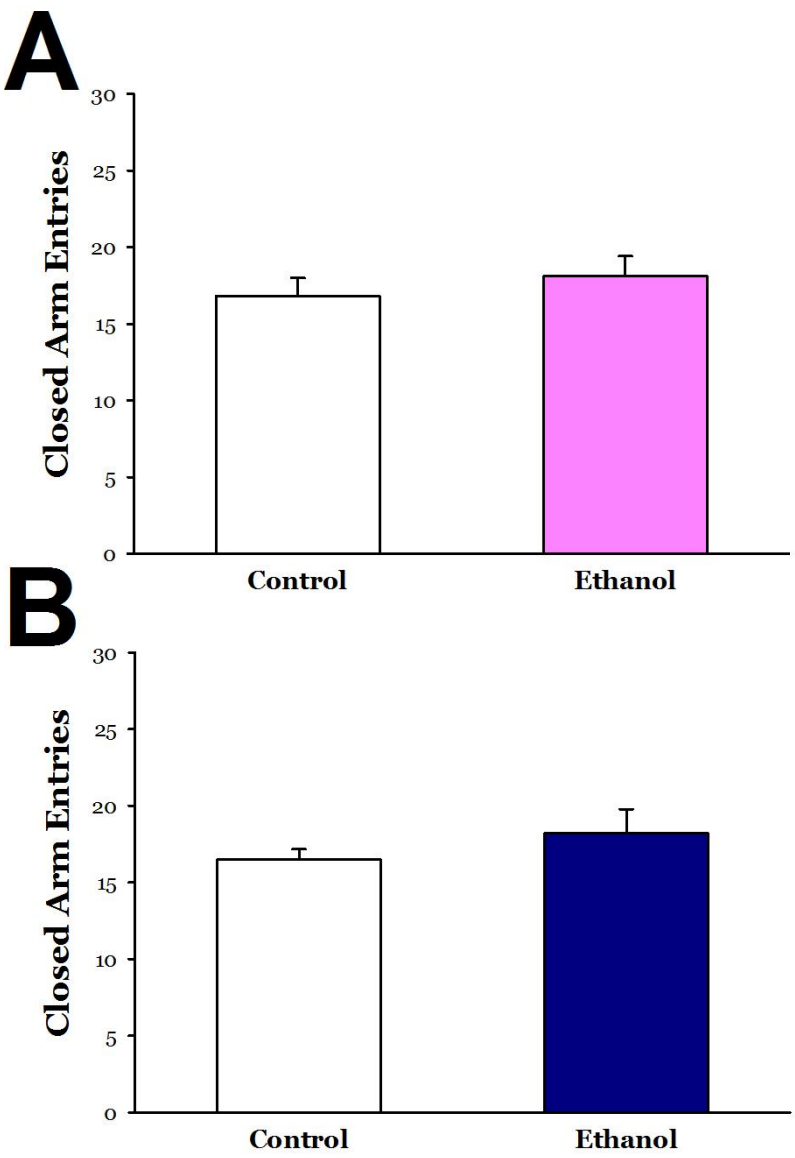
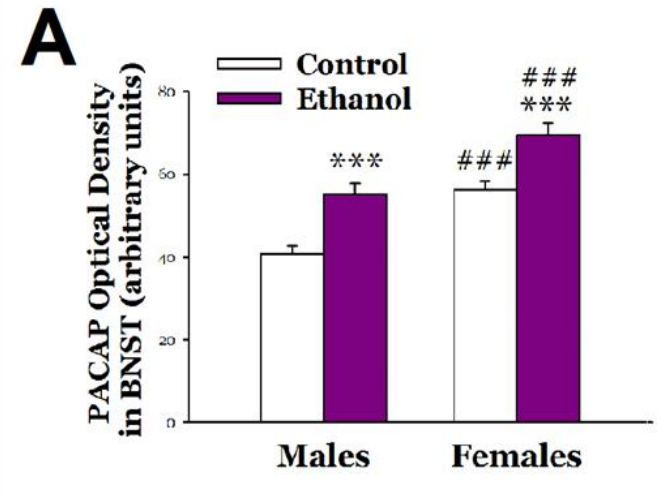


Fig. 8. Number of entries into closed arms of the EPM. A) Female mice control vs. ethanol drinking females had a similar number of closed arm entries. B) Male control mice vs. ethanol drinking males had a similar number of closed arm entries. Data are Mean+SEM.

Immunohistochemistry

As shown in Fig. 9, animals exposed to chronic, intermittent ethanol showed a significant increase in their PACAP expression in the BNST area, compared with their control counterpart (Group: $F(1,19)= 34.39$; $p< 0.001$; Sex*Group: $F(1,19)= 0.06$, n.s.). Female mice exhibited a markedly higher BNST PACAP expression compared to males (Sex: $F(1,19)= 40.14$; $p< 0.001$).

Fig. 9



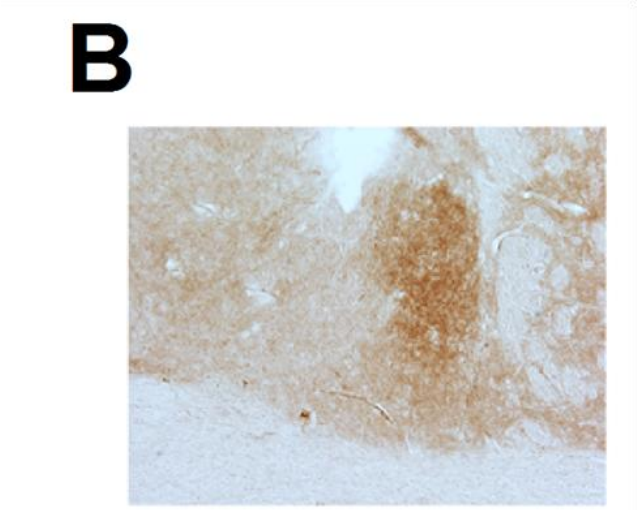


Fig. 9 PACAP levels in the BNST of control and ethanol drinking C57Bl/6 male and female mice. A) PACAP levels were elevated in the BNST of mice exposed to chronic, intermittent access to alcohol, compared to Controls *(N* = 5-8/group). B) Representative PACAP staining in BNST of mice (10x). Data are Mean +SEM. *** p <0.001 vs. Control; ### $p < 0.001$ vs. male Control.

DISCUSSION

These experiments support the notion that chronic, intermittent alcohol drinking recruits the BNST PACAP system in mice. Female mice showed higher intake and preference for alcohol compared to male mice in this chronic, intermittent ethanol, two-bottle choice paradigm. We chose the intermittent ethanol access two bottle choice paradigm, because it has been shown to produce escalation of intake, high levels of ethanol consumption and preference over a long period of time (Crabbe et al. 2012; Simms et al. 2008). This paradigm also consists of repeated binge/intoxication and withdrawal cycles through voluntary ethanol intake, as opposed to ethanol vapor exposure, which elicits dependence but being experiment-administered does not follow the human patterns of alcohol intake. This experiment demonstrated that female mice show a higher preference for and intake of alcohol than their male counterparts in the two-bottle choice paradigm. Female mice reached average alcohol intake levels of 23-24 g/kg compared to 12-14 g/kg in male mice.

Other studies have similarly demonstrated the sexual dimorphisms in alcohol consumption; one such study found that female rats of many different breeds maintained greater alcohol intake than their male counterparts (Li and Lumeng 1984; Piza-Palma et al. 2014). Studies have investigated the role of gender in alcohol consumption through ovariectomization and supplementation with estradiol and progesterone (Almeida et al. 1998). Ovariectomized females display reduced ethanol intake compared to intact females, though still higher than males (Almeida et al. 1998).

It should be noted that while female animals show a higher prevalence of alcohol intake than their male counterparts in a variety of models, humans seem to show the opposite trend. Men are more likely to drink alcohol and more likely to binge drink, though that gap is quickly closing (Keyes et al. 2010). On the other hand, women progress from alcohol use to addiction more quickly, which is termed "telescoping" (Brady and Randall 1999). This suggests that women could be more vulnerable to chronic alcohol consumption than men.

Several studies recently focused on the gender-related impairment of the central nervous system (Hommer et al. 2001; Pfefferbaum et al. 2001). Despite fewer years of heavy drinking than men, the degree of cognitive dysfunction in alcoholic women is similar to that in alcoholic men (Nixon and Glenn 1995). In addition, there are studies that report similar expansion of intracranial cerebrospinal fluid (CSF) volume among male and female alcoholics as opposed to control subjects, though female subjects had shorter exposure time to excessive drinking (Hommer et al. 2001; Jacobson 1986; Mann et al. 1992) with the use of magnetic resonance imaging showed that the brain volume differences between alcoholic and nonalcoholic women were significantly higher than those between alcoholic and nonalcoholic men, regardless of female alcoholics reporting less years of heavy drinking (Hommer et al. 2001). This suggests that woman may be more vulnerable to the aversive effects of alcohol.

Anxiety-like behavioral tests showed unpredicted results. Analyzing with a two-way ANOVA saw an effect of sex in both latency and time spent in the withdrawal

chamber. We hypothesized that we would an effect of group on the latency and time spent in the chamber because the mice were tested during a withdrawal period. Female mice had a shorter duration of time in the withdrawal chamber and a shorter latency to enter open arena. We did, however, observe an effect of group in transitions and average time spent in the open field each visit. The effect of group was seen in male mice for transitions; female mice showed no significant differences in transitions. Ethanol drinking male mice had significantly less transitions than control mice, which could reflect either an effect on anxiety-like behavior or on general motor activity. Regarding average time spent in the open arena, the effect of ethanol was observed in the male mice. In regard to our EPM, there was an effect of sex*group in percentage of total time on open arms. Male controls spent significantly less percent of time on the open arms than ethanol drinking. We would expect to see the opposite as less time in the open arms is usually a sign of anxiety-like behavior. Male control mice also spent significantly less percent of time in the open arms compared to females.

Latency and time in the withdrawal chamber in defensive withdrawal, as well as time spent on the open arms on the EPM are normal indicators anxiety-like behavior in a subject. Many studies on the sex differences in ethanol use have shown that male subjects generally show more anxiety-like behaviors in the EPM (Overstreet et al. 2004) as well as social interaction test (Varlinskaya and Spear 2004). In addition, female subjects show a lower proclivity to withdrawal, which may be due to the elevated activity of progesterone and endogenous steroids (Tanchuck-Nipper et al. 2015). Less intense withdrawal symptoms have also been associated with enhanced progesterone and

allopregnanolone levels (Martin-Garcia and Pallares 2005). There are also neurochemical differences in male and female animal subjects that could account for the discrepancy in withdrawal symptoms, such as the increase of GABA alpha-1 receptor in the male rat cerebral cortex (Devaud et al. 1997) or the variations of the GluN2B subunit of the NMDA receptor in the cortex (Devaud and Morrow 1999).

We hypothesize that the expected lack of anxiety-like behavior observed in ethanol-drinking mice may be a consequence of the time point chosen for the observation. Indeed, these tests were performed 24 hr after the end of the previous ethanol access, while it is conceivable that anxiety-like behavior may be present at a shorter, more acute withdrawal time point in this model. Future experiments will test this hypothesis.

These experiments also demonstrate that excessive intermittent alcohol consumption causes a marked increase in PACAP immunoreactivity in the oval nucleus of the BNST of mice. Indeed, both male and female mice exposed to intermittent ethanol access displayed significantly higher levels of PACAP compared to their relative control. In addition, PACAP immunoreactivity in control female mice was significantly higher compared to male control mice.

The BNST is an important structure in the stress response and is hypothesized to have an important role in AUDs. It is responsible for the synthesizing of mood and negative valence information and additionally sustaining anxiety-like behavior; the oval nucleus of the BNST receives CRF, GABA, PACAP, dopamine, and encephalin signals

(Kim et al. 2013). The oval nucleus sends GABAergic projections to the CeA, VTA, and lateral hypothalamus (Dong et al. 2001b). CeA GABAergic neurons, some co-expressing CRF (Forray and Gysling 2004), reciprocally project to the oval nucleus of the BNST (Daniel and Rainnie 2016). Chronic stress paradigms and foot shock enhance CRF mRNA levels in the oval nucleus (Daniel and Rainnie 2016), as well as an upregulation of PACAP by more than 10 fold and its receptor PAC1 by 2 fold, in the oval nucleus of the BNST, which have been found to engage with CRF-positive neurons (Hammack et al. 2009; Hammack et al. 2010; Kozicz et al. 1997).

The next steps of research would be to test the functional role of the PACAP increase by administering either PACAP receptor antagonists or an shRNA to knock the receptor down directly into the oval nucleus of the BNST, and test their effects on excessive alcohol consumption and anxiety-like behavior in mice subject to this drinking paradigm. This research, therefore, lays the ground to more extensive work to unravel the neurobiology of excessive drinking.

LIST OF JOURNAL ABBREVIATIONS

Behav Brain Res	Behavioral Brain Research
Bio Psychiatry	Biological Psychiatry
Annu Rev Psychol	Annual Review Psychology

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