Binghamton University
The Open Repository @ Binghamton (The ORB)

Undergraduate Honors Theses | Dissertations, Theses and Capstones

Spring 5-2023

Effects of Acute and Repeated Alcohol Exposure on Expression of Synaptic-Associated Genes in the Male and Female Mouse mPFC

Dhruba Podder
Binghamton University--SUNY, dpodder1@binghamton.edu

Follow this and additional works at: https://orb.binghamton.edu/undergrad_honors_theses

 Part of the Behavioral Neurobiology Commons, Cognitive Neuroscience Commons, and the Molecular and Cellular Neuroscience Commons

Recommended Citation

Podder, Dhruba, "Effects of Acute and Repeated Alcohol Exposure on Expression of Synaptic-Associated Genes in the Male and Female Mouse mPFC" (2023). *Undergraduate Honors Theses*. 31.
https://orb.binghamton.edu/undergrad_honors_theses/31

This Thesis is brought to you for free and open access by the Dissertations, Theses and Capstones at The Open Repository @ Binghamton (The ORB). It has been accepted for inclusion in Undergraduate Honors Theses by an authorized administrator of The Open Repository @ Binghamton (The ORB). For more information, please contact ORB@binghamton.edu.

Effects of Acute and Repeated Alcohol Exposure on Expression of Synaptic-Associated Genes in the Male and Female Mouse mPFC

Honors Thesis

Integrative Neuroscience

Spring 2023

Dhruba Podder

Department of Psychology

Binghamton University

State University of New York (SUNY)

Honors Thesis Committee:

Dr. Florence Varodayan (Chair)

Dr. Marvin Diaz

Dr. Joshua Peck

© Copyright by Dhruba Podder 2023

All Rights Reserved

Abstract

Alcohol use disorder (AUD) is a serious chronic brain disease; in 2021 there were ~29.5 million people in the U.S. diagnosed with AUD. Individuals with AUD often show cognitive impairment such as risky decision-making, difficulties with impulse control, and working memory deficits. This impairment is associated with structural and functional changes in the prefrontal cortex (PFC), an extensively interconnected region of the frontal lobe involved in executive control of goal-directed behaviors. Chronic alcohol exposure in rodents has been seen to cause deficits in performance in behavioral tasks which assess mPFC function such as working memory and behavioral flexibility. It is thought that the remodeling of mPFC synapses contributes to alcohol-related cognitive impairment, but it is not known which specific pathways and genes are responsible for alcohol's long-term effects. Given that synaptic transmission is likely an important contributor to the development of these long-term cognitive deficits, it is surprising that relatively little is known about how alcohol affects expression of the synaptic vesicle fusion machinery in males and females. Thus, the purpose of this study was to investigate how acute and repeated alcohol exposure modify the gene expression of synaptic vesicle-associated proteins, including HSF1, VAMP1, VAMP2, and Syt1 in the mPFC of male and female mice Through qPCR, we found significant sex and alcohol exposure differences in synaptic gene expression. In males, *Vamp1* expression increased after acute alcohol exposure and decreased after repeated alcohol exposure. In females, *Vamp2* expression increased after acute and repeated ethanol exposure and *Hsf1* expression increased after repeated ethanol exposure. These findings suggest that ethanol differentially induces changes in the expression of synaptic genes in male and female mPFC, which likely impacts neurotransmission through changes in the synaptic vesicle fusion machinery. Additionally, this suggests that there is a compensatory mechanism to

counteract the effects of multiple ethanol exposures, which is present in the male mPFC but not in the female mPFC. Therefore, future studies should investigate potential sex differences through a systems biology approach by investigating how alcohol can regulate expression and function of postsynaptic GABA and glutamate receptors, as well as GABA and glutamate release.

Table of Contents

List of Tables

List of Figures

Introduction

Alcohol use disorder (AUD) is a serious chronic brain disease; in 2021 there were ~29.5 million people in the U.S. diagnosed with AUD (Substance Abuse and Mental Health Services Administration [SAMHSA], 2022). Between 2002 and 2019, the annual percentage of people with AUD had been steadily declining, but between 2019 to 2021, this percentage doubled from 5.3% to 10.6%. Binge drinking is considered a pattern of alcohol consumption which is characterized by drinking to a blood alcohol concentration (BAC) of at least 0.08%, which in humans is equivalent to 5 drinks in men and 4 drinks in women in a period of 2 hours (Jones et al., 2018). Individuals with AUD who engage in binge drinking often show cognitive impairment such as risky decision-making, difficulties with impulse control, and working memory deficits (Bernardin et al., 2014).

Neuroimaging studies provide a way to investigate how alcohol exposure in humans may impact the structure and connectivity of various brain regions. One particular study demonstrated that greater reported frequency and quantity of alcohol consumption were associated with altered frontal lobe gray-matter volumes (Kvamme et al., 2016). These changes were sexually dimorphic, such that binge drinking men had smaller volumes than healthy volunteer males while binge drinking women had larger volumes than their female counterparts. Further sex differences were noted in another study investigating cortical thickness, where binge drinking men had thinner cortices than non-drinker men while binge drinking women had thicker cortices than non-drinker women (Squeglia et al., 2012).

The human prefrontal cortex (PFC) is an extensively interconnected region of the frontal lobe involved in executive control of goal-directed behaviors (Murty et al., 2017). PFC function is particularly vulnerable to the effects of alcohol, and chronic alcohol can cause behavioral

disinhibition which is associated with deficits in decision making, response inhibition, social anxiety and working memory (Li et al., 2021). The ventromedial and dorsolateral parts of the PFC play regulatory roles in various cognitive functions including attention, inhibitory control, habit formation, and working, spatial or long-term memory (Jobson et al., 2021). The hallmark cognitive impairment exhibited by AUD patients is associated with structural and functional changes in the mPFC, exemplified through neuroimaging studies in humans (Koob and Volkow, 2016). For example, after alcohol use, smaller ventromedial PFC volumes were seen in binge drinking male and female adults compared to naïve controls (Campanella et al., 2013). With regards to the functional changes induced by alcohol, higher consumption of alcohol per drinking occasion in binge drinking male and female adults were associated with greater activation in the dorsolateral PFC (Jones et al., 2018).

The rodent medial prefrontal cortex (mPFC) is functionally homologous to the ventromedial and dorsolateral regions of the human PFC, with the two sharing a similar structure and function (Anastasiades and Carter, 2021). Chronic alcohol exposure in rodents has been seen to cause deficits in performance in various behavioral tasks including the Barnes maze, Morris water maze, novel object recognition, and social interaction tasks (Macht et al., 2020). These chronic ethanol-induced behavioral deficits in rodents have been seen to correspond to impaired working memory, reduced behavioral flexibility, and increased impulsivity (Charlton et al., 2019). While most *in vivo* rodent alcohol studies were performed only using male subjects, which limits our understanding of the underlying pathways, there are a few studies that have explored both sexes. A study found that chronic ethanol exposure leads to decreased expression of $\alpha 2$ subunits of $GABA_A$ receptors in the VTA, NAc, BNST, and CeA in female mice, but not male mice (Logrip et al., 2017). Another study which investigated the effects of adolescent alcohol exposure on

myelination found that male rodents are more sensitive than females to the effects of alcohol administration on myelinated axons and myelin genes in the PFC (Tavares et al., 2019). Preliminary findings from the Varodayan lab have also shown that mice that are exposed to chronic alcohol through a voluntary drinking paradigm show deficits in long-term reference memory compared to water controls using a modified Barnes maze task. Interestingly, there was a sex difference in cognitive function, with males showing greater deficits compared to the female mice regardless of their drinking solution (data unpublished).

The synapses of the mPFC are highly sensitive to alcohol, and it is thought that their remodeling contributes to alcohol-related cognitive impairment. In general, synaptic remodeling requires increased expression of genes related to neurotransmitter release and the postsynaptic receptors that these neurotransmitters bind. Alcohol ubiquitously induces changes in many genes (Ferguson et al., 2022; Worst & Vrana, 2005), including those encoding synaptosomal-associated protein 25 kDa (SNAP-25), Rab3, Munc18, VAMP1, VAMP2, and Synaptotagmin 1 (Das, 2020), but it is not known which specific pathways and genes in the mPFC are responsible for alcohol's long-term effects on cognitive function. The process of neurotransmission requires the release of neurotransmitters from synaptic vesicles through a process of membrane fusion with the presynaptic membrane (Martens and McMahon, 2008; Yang et al., 2017). Synaptotagmin 1 (Syt1), along with soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), make up a complex protein machinery which coordinates this membrane fusion process, leading to neurotransmitter release (Jahn & Scheller, 2006). Syt1 is one protein of particular interest as it acts as the calcium sensor to directly trigger synaptic vesicle fusion. In a previous *in vitro* study using mixed-sex primary cortical culture, *Syt1* gene expression was upregulated by 1 hour of 60

mM alcohol exposure (Varodayan et al., 2011) via activation of the transcription factor heat shock factor 1 (HSF1).

Vesicle associated membrane proteins (VAMP, also known as synaptobrevins), help position synaptic vesicles on the presynaptic terminal, so they are ready for fusion (Martens & McMahon, 2008). VAMP2 is the more abundant isoform compared to VAMP1 and is more widely distributed throughout the brain (Raptis et al., 2005). In a previous study using mouse primary cortical cultures, ethanol increased *Vamp2* mRNA levels, but did not alter *Vamp1* (Varodayan et al., 2011). This same study showed that ethanol's effects on *Vamp2* gene expression were concentration-dependent, such that *Vamp2* transcription was induced more strongly by higher ethanol concentrations. It was also shown that HSF1 activity mediates ethanol induction of *Vamp2* gene expression (Varodayan & Harrison, 2013). The cortical cultures used in these studies were not stratified by sex, so was not known what role sex may play in the expression of these synaptic genes or whether these specific genes would also be induced by *in vivo* alcohol exposure.

Given that synaptic transmission is likely an important contributor to the development of alcohol-induced cognitive deficits, it is surprising that relatively little is known about how alcohol affects expression of the synaptic vesicle fusion machinery in males and females. Therefore, the purpose of this study was to investigate how acute and repeated alcohol exposure modify the gene expression of various synaptic vesicle-associated proteins, including HSF1, VAMP1, VAMP2, and Syt1 in the mPFC of mice. The secondary purpose of the present study was to determine whether sex had an effect on the magnitude or direction of any potential change in synaptic vesicle-associated gene expression induced by alcohol. We hypothesized that *in vivo* acute alcohol would increase *Vamp2* and *Hsf1* mRNA levels in the mPFC compared to alcohol-naïve controls, similar to the previous *in vitro* studies. Following the ethanol concentration-dependent gene expression

changes seen in a past *in vitro* study (Varodayan & Harrison, 2013), we predicted that the effects of *in vivo* repeated alcohol on synaptic vesicle-associated gene expression would be greater compared to acute alcohol due to the alcohol-induced changes accumulating over repeated ethanol exposure. From a number of past studies, there are several sex differences that can be seen in ethanol's effects on behavior, cognitive function, and gene expression, but the directions of these sex-dependent changes have not been fully elucidated. Thus, it was hypothesized that there would be sex differences present in ethanol-induced gene expression, but we were unsure of which direction they would be seen in.

Methods

Subjects

Adult male and female C57BL/6J mice were obtained from Jackson Labs (Bar Harbor ME) at ages 10-12 weeks (N=35 per sex). Mice were acclimated to the colony room and handled for one week. All animals were housed in groups of three in standard cages in a temperature-and humidity-controlled room and had access to food and water *ad libitum*. The colony room which the mice were housed in for the duration of the experiments was on a reverse light cycle (lights on at 10 PM and lights off at 10 AM). For all experiments including female mice, estrous cycle samples were collected from the mice immediately after sacrifice. For all animals receiving ethanol treatment through intraperitoneal (i.p.) injection, blood was collected immediately after decapitation for blood ethanol concentration (BEC) determination. All procedures were approved by the SUNY Binghamton University Institutional Animal Care and Use Committee and conformed to federal regulations and National Institutes of Health guidelines for the use and care of laboratory animals.

Intraperitoneal (i.p.) injections

Mice were transported to the procedure room and habituated for 4 hours per day, from 10 AM to 2 PM, for three days prior to the start of i.p. injections. After the weekend off, mice were transported to the procedure room and received an i.p. injection 1 hour later for five consecutive days. Control mice received physiological saline for all five days, acute exposure mice received physiological saline for the first four days and ethanol (2.5 g ethanol in saline/kg body weight) on day five, and repeated exposure mice received ethanol (2.5 g/kg) for all five days. All treatment conditions were counterbalanced within each cohort. A five-day timeline of treatment conditions and groups can be seen in **Figure 1.**

Blood ethanol concentrations

Blood was collected at sacrifice and the samples were centrifuged for 20 min at 13000 rpm at 4 ºC. The supernatant was stored at –80 ºC, and then processed in a GM7 analyzer (Analox Instruments, London, UK) according to the manufacturer's instructions.

Gene expression

90 to 120 minutes after the final i.p. injection, mice were anesthetized with 3-5% isoflurane. Their brains were extracted and immediately flash frozen using 2-methylbutane and placed on dry ice until stored at –80 ºC. Brains were sliced using a cryostat (maintained at –18 ºC) and bilateral mPFC tissue micropunches (0.75 mm) were collected in 2 mL Eppendorf tubes and stored in a –80 ºC freezer. The samples were homogenized in Trizol reagent (Sigma-Aldrich, St. Louis, MO) with 5 mm stainless steel beads (Qiagen, Germantown, MD) and a TissueLyser II machine (Qiagen). Total RNA was then extracted using RNeasy columns (Qiagen) according to

manufacturer instructions. Total RNA concentration and purity was quantified via a Nanodrop spectrophotometer (Themoscientific, Waltham, MA). cDNA synthesis was performed using the QuantiTect Reverse Transcription kit (Cat. No. 205313, Qiagen). Real time-polymerase chain reaction (rt-PCR) was performed using the CFX384 real-time PCR detection system, the IQ SYBER Green Supermix (Biorad, Hercules, CA) and cDNA template and primers for the following genes: *Hsf1*, *Syt1*, *Vamp1*, *Vamp2* and *Actb*. A list of forward and reverse primers used in this study can be found in **Table 1**. A single peak in the melt curve indicates primer pair specificity for the target gene.

Statistical Analyses

Statistical analyses were performed using Prism (GraphPad, San Diego, CA), with differences significant at $p < 0.05$. All data is represented as mean ± SEM. Final blood ethanol concentration (BEC) values for males vs. females were analyzed using unpaired *t*-tests. Gene expression data was normalized to *Actb* (**Figure 3)** and quantified as percent change from control mice, with the male and female control data designated as ultimate controls. The final values for each gene were analyzed using a one-way ANOVA. *Post hoc* Tukey's comparisons tests were used where appropriate. Pearson correlations were also run between BECs and normalized gene expression.

Results

Blood ethanol concentrations

Unpaired *t*-tests were performed to analyze the effect of EtOH treatment condition on BEC in males **(Figure 2A)** and females **(Figure 2B)**. There were no statistically significant differences

in BEC between acute and repeated EtOH-exposed males, or between acute and repeated EtOH-exposed females.

Molecular studies

We explored mPFC synaptic-associated protein gene expression in males and females using rt-PCR and one-way ANOVA tests to compare the effect of EtOH treatment conditions on mRNA levels of the genes encoding for the synaptic-associated proteins, HSF1, VAMP1, VAMP2, and Syt1. Importantly, there was no significant difference in the reference gene *Actb* mRNA levels across ethanol treatment in either the male **(Figure 3A)** or female mPFC **(Figure 3B)**. A summary of all rt-PCR one-way ANOVA statistical outcomes can be found in **Table 2.**

There was no statistically significant difference between treatment conditions in the mean mRNA levels of the genes encoding for HSF1, Syt1, and VAMP2 in the mPFC of males (**Figure 4A, B, D)**. There was a statistically significant difference between treatment conditions in the mean mRNA levels of the gene encoding for VAMP1 in the mPFC of males **(Figure 4C)**. *Post-hoc* Tukey's multiple comparisons test found that the mean *Vamp1* mRNA levels were significantly higher in acute EtOH-treated males compared to control males and repeated EtOH-treated males.

There was no statistically significant difference between treatment conditions in the mean mRNA levels of the genes encoding for Syt1 and VAMP1 in the mPFC of females (**Figure 5B, C)**. There was a statistically significant difference between treatment conditions in the mean mRNA levels of the gene encoding for HSF1 in the mPFC of females **(Figure 5A)**. *Post-hoc* Tukey's multiple comparisons test found that the mean *Hsf1* mRNA levels were significantly higher in repeated EtOH-treated females compared to control females. There was also a statistically significant difference between treatment conditions in the mean mRNA levels of the

gene encoding for VAMP2 in the mPFC of females **(Figure 5D)**. *Post-hoc* Tukey's multiple comparisons test found that the mean *Vamp2* mRNA levels were significantly higher in acute EtOH-treated females and repeated EtOH-treated females compared to control females.

Thus, we found significant differences in *Vamp1* mRNA levels between treatment conditions in the male mPFC and found significant differences in *Hsf1* and *Vamp2* mRNA levels between treatment groups in the female mPFC.

BEC – gene expression correlations

A Pearson correlation coefficient was computed for all male **(Figure 6A)** and female **(Figure 6B)** EtOH-treated mice to assess the linear relationship between BEC and gene expression of *Hsf1*, *Vamp1*, *Vamp2*, and *Syt1* quantified as percent change from control. A summary of all BEC – normalized gene expression correlational statistical outcomes can be found in **Table 3.** There were no statistically significant correlations between BEC and expression of *Hsf1*, *Vamp1*, *Vamp2*, and *Syt1* in EtOH-treated males and females.

Discussion

It has been previously established that synaptic remodeling through alcohol-induced changes in expression of neurotransmission-associated genes contributes to structural and functional changes in the mPFC. However, our understanding of the underlying pathways and the role that sex may play is limited by the vast majority of *in vivo* rodent alcohol studies having been performed in only male subjects. Thus, in the present study, we investigated how acute and repeated EtOH exposure modified the expression of genes encoding various vesicle-associated proteins, including HSF1, VAMP1, VAMP2, and Syt1 in the mPFC of male and female mice. We

found that in males, there were significant differences in *Vamp1* expression between the acute EtOH treatment group and the control and repeated EtOH treatment groups, but in females, there were significant differences in *Hsf1* and *Vamp2* expression between the acute and repeated EtOH treatment groups and the control group.

Sex differences in ethanol's effects on structural plasticity

The results of our study suggest that there are sex-dependent effects of ethanol exposure on gene expression in the mPFC. In particular, it was found that in males, *Vamp1* was significantly induced by ethanol exposure, but in females, *Hsf1* and *Vamp2* were significantly induced by ethanol exposure. The sex differences observed here may be explained by differences in the way that ethanol impacts the mPFC of males and females on the neurocircuitry level and down to the molecular level.

It is widely understood that in both rodents and humans, there are sex differences in the pharmacokinetic properties of ethanol (Cortez et al., 2020), including absorption, distribution, metabolism, and elimination, as well as in the behavioral and functional effects of ethanol (Flores-Bonilla & Richardson, 2020). Although sex differences in pharmacokinetics of ethanol likely plays a large role in ethanol's effects on cortical structure and function, it does not fully explain the differential changes in structural plasticity in males and females.

Gonadal steroid hormones play an important role in modulating various functions in the brain including synaptic plasticity (Hyer et al., 2018) and cognition, memory, and learning driven by the hippocampus and the PFC (Hamson et al., 2016). These regulatory roles of gonadal steroid hormones such as estradiol, progesterone, and testosterone may in part explain the sex differences in the ethanol-induced structural and functional changes in the brain (Flores-Bonilla & Richardson,

2020). The presence of different concentrations of the different gonadal steroid hormones may explain why genes are differentially induced in the male and female mPFC as a result of ethanol exposure.

It has been established that ethanol, itself, is considered to be a stressor, which activates the hypothalamic-pituitary-adrenocortical (HPA) axis, which is a major component of the neuroendocrine stress response (Becker, 2017). Structural plasticity in neurons is described by multiple dimensions including neuron structure, dendritic arborization and spine density (Garrett & Wellman, 2009). Alterations in parameters of structural plasticity such as dendritic arborization and spine density are induced by stress and these alterations are different in males and females. For example, it has been seen that in the PFC, chronic stress leads to increased length and complexity of dendritic arbors in females but leads to a reduction in dendritic arbors in males (Farrell et al., 2015). Thus, it is possible the stress induced by acute and repeated ethanol exposure can differentially impact structural plasticity in the male and female mPFC, which in turn induces expression of different synaptic genes.

Effect of acute and repeated ethanol exposure on synaptic-associated gene expression

We found that in the male mPFC, there was a significant increase in *Vamp1* expression between the control and acute ethanol-treated groups and that there was a significant decrease in *Vamp1* expression between the acute and chronic ethanol-treated groups. This finding in males suggests that there may be a compensatory mechanism to bring gene expression back to a baseline which lead to neuroadaptations as a result of multiple ethanol exposures. In the female mPFC, there was a significant increase in *Hsf1* expression between the control and repeated ethanol-treated groups. There was also a significant increase in *Vamp2* expression between the control

group and the acute and repeated ethanol-treated groups. These observed changes in gene expression in the female mPFC may indicate that females do not have the same kind of compensatory mechanism as seen in the male subjects since there was no indication of a return to baseline gene expression after multiple ethanol exposures.

Additionally, it is interesting that in males, only *Vamp1* was induced by ethanol while in females, *Vamp2* and *Hsf1*, but not *Vamp1* was induced by ethanol. This is particularly notable because in a past *in vitro* study, it was found that in a mixed-sex primary cortical culture, *Vamp2* expression was induced by ethanol and HSF1 activity mediated this induction of *Vamp2* (Varodayan & Harrison, 2013). The effect of ethanol in the female mPFC correlates with this past finding, but the male results show no ethanol-induced increases in *Hsf1* or *Vamp2* expression. This can potentially be explained by different pathways being activated in the male and female mPFC following ethanol exposure.

The theory of a compensatory mechanism associated with multiple ethanol exposures can be supported by a rebounding effect in the degree of negative affect and in stress hormone levels which can be seen during the withdrawal phase after the effects of ethanol wear off (Peltier et al., 2019). Furthermore, this rebounding effect during the withdrawal phase differs with sex.

Sex and ethanol exposure-dependent differences in BEC

We found that in males, there was no difference in BEC between acute and repeated ethanol-exposed mice, but in females, there was an increase in BEC between acute and repeated ethanol-exposed mice. Parallel to the potential compensatory mechanism seen in mPFC expression of *Vamp1* between acute and repeated ethanol-exposed males, it is possible that this mechanism is able to induce neuroadaptations which result from protracted ethanol exposure. Furthermore, as

this compensatory mechanism was not seen in mPFC expression of *Hsf1* and *Vamp2* between acute and repeated ethanol-exposed females, it is possible that a lack of this mechanism leads to an increased induction of BEC and decreased rate of ethanol metabolism in females with multiple ethanol exposures.

Limitations

Although the present study included both male and female subjects to investigate the impact of sex on ethanol-induced changes in synaptic vesicle fusion machinery, there are a number of limitations of the methods and design of this study. Firstly, the male and female subjects were run as separate cohorts which means that the gene expression and BEC data for each cannot be directly compared between males and females. Due to this, any potential interaction between sex and EtOH treatment on BEC or gene expression cannot be definitively explored. Another limitation of this study is that the use of an *in vivo* model led to an inability to consistently induce clinically relevant BECs over limited periods of time in subjects as compared to *in vitro* models of EtOH exposure. In previous *in vitro* studies, cortical neurons were exposed to specific concentrations of EtOH over specific time periods by adding EtOH directly to the culture medium (Varodayan et al., 2011; Varodayan & Harrison, 2013). In this study, we only measured gene expression and did not directly investigate the resulting protein levels or functional changes in neurotransmission. Due to this, we are unsure if the changes seen in synaptic-associated gene expression correlate with changes in protein concentration as measured by western blot or changes in neurotransmitter tissue content as measured by HPLC and microdialysis or changes in EPSP- or IPSP-spike potentiation as measured by whole-cell patch-clamp electrophysiology. Lastly, due to *in vivo* differences in ethanol metabolism between subjects and between sexes, as seen in the

between-subject and between-sex variability in **Figures 2 and 6**, potential correlation between BEC and gene expression could not be directly explored. Despite this, it is expected that higher concentrations of EtOH would induce higher gene expression, since this concentration-dependent relationship was seen with *Syt1* expression *in vitro* (Varodayan et al., 2011).

Conclusions

The goal of the present study was to investigate how different levels of ethanol exposure in male and female mice affect the synaptic vesicle fusion machinery which contains several proteins critical to neurotransmission. We found a key sex difference in gene expression such that in males, there was a significant ethanol-induced increase in *Vamp1* expression, but in females, there was a significant ethanol-induced increase in *Hsf1* and *Vamp2*, but not *Vamp1*. Furthermore, it was found that there may be a compensatory mechanism to induce neuroadaptations as a result of multiple ethanol exposures in males, but not in females. Interestingly, it was found that in both the male and female mPFC, there was no significant difference in *Syt1* expression between ethanol exposure groups. This finding does not support the previous finding that *Syt1* expression is induced by ethanol exposure (Varodayan et al., 2011; Varodayan & Harrison, 2013).

Given that the present study only investigated the effects of ethanol on gene expression, future studies could examine the functional consequences of the ethanol-induced synaptic-associated gene expression changes shown here. For example, we could examine synaptic protein levels via western blot, neurotransmitter tissue content via HPLC and microdialysis, or functional changes in neurotransmission via electrophysiology. Synaptic changes like those seen resulting from ethanol exposure contribute to changes in the machinery by which neurotransmitters are released into the synapse. Following these changes, it is expected that the levels of the proteins

these genes encode would exhibit a change in the same direction, which would modulate the probability and frequency of neurotransmitter release. Given the nature of ethanol's actions as a CNS depressant, it is likely that the resulting functional changes in GABAergic and glutamatergic neurotransmission would not be seen in the same direction or perhaps even would exhibit changes in only one of the two. These kinds of functional changes in the rodent mPFC likely correspond to an increase in ethanol withdrawal-associated behaviors including anxiety-like behaviors, increased pain sensitivity, escalation of ethanol consumption, deficits in cognitive function such as memory and learning, and interruptions in slow wave sleep and latency to sleep (Huitron-Resendiz et al., 2018; Patel et al., 2021). It can be inferred that in humans, the long-term changes in mPFC synapses potentiated by chronic ethanol exposure likely lead to increases in withdrawal behaviors and craving over time. Thus, these molecular level changes may contribute to the development of alcohol use disorder through maintenance and escalation of binge drinking alongside behaviors such as craving, withdrawal, and aspects of negative affect including feelings of stress and anxiety (Flores-Bonilla & Richardson, 2020).

Following the sex differences seen in both gene expression and BEC induction by ethanol, future studies could also investigate the underlying mechanisms of the sex-dependent effects observed in our study. Since the HPA axis and gonadal steroid hormones play such an important role in structural plasticity, their contribution to ethanol-induced changes in neurotransmission should be explored. Finally, following the potential compensatory mechanism seen in the male mPFC, but not the female mPFC, it would be interesting to investigate whether the changes in synaptic-associated gene expression observed in this study are reversible or persistent. One potential route to explore this is by investigating whether inactivating HSF1 transcriptional activity would reverse the changes that are seen in *Vamp1* or *Vamp2* expression. Alternatively, we could

explore a time course of changes in gene expression following ethanol exposure and investigate whether these changes persist after the cessation of ethanol exposure. To explore the ways in which ethanol's effects on gene expression may translate to withdrawal-associated behaviors in rodents, we could assess the differences in behavior of ethanol exposed female mice before and after inactivation of HSF1 transcriptional activity *in vivo* by using behavioral paradigms including Barnes maze and elevated plus maze. Overall, this study demonstrated that ethanol acts in a sex and exposure-dependent manner and differentially impacts synaptic gene expression in the mPFC.

References

Abrahao, K. P., Salinas, A. G., & Lovinger, D. M. (2017). Alcohol and the Brain: Neuronal Molecular Targets, Synapses, and Circuits. *Neuron*, *96*(6), 1223–1238. (Varodayan et al., 2011; Varodayan & Harrison, 2013)

Anastasiades, P. G., & Carter, A. G. (2021). Circuit organization of the rodent medial prefrontal cortex. *Trends in Neurosciences*, *44*(7), 550–563. https://doi.org/10.1016/j.tins.2021.03.006

Apkarian, A. V., Neugebauer, V., Koob, G., Edwards, S., Levine, J. D., Ferrari, L., Egli, M., & Regunathan, S. (2013). Neural mechanisms of pain and alcohol dependence. *Pharmacology, Biochemistry, and Behavior*, *112*, 34–41. https://doi.org/10.1016/j.pbb.2013.09.008

Becker, H. C. (2017). Influence of Stress Associated with Chronic Alcohol Exposure on Drinking. *Neuropharmacology*, *122*, 115–126. https://doi.org/10.1016/j.neuropharm.2017.04.028

Bernardin, F., Maheut-Bosser, A., & Paille, F. (2014). Cognitive Impairments in Alcohol-Dependent Subjects. *Frontiers in Psychiatry*, *5*. https://www.frontiersin.org/articles/10.3389/fpsyt.2014.00078

Campanella, S., Peigneux, P., Petit, G., Lallemand, F., Saeremans, M., Noël, X., Metens, T., Nouali, M., De Tiège, X., De Witte, P., Ward, R., & Verbanck, P. (2013). Increased cortical activity in binge drinkers during working memory task: A preliminary assessment through a functional magnetic resonance imaging study. *PloS One*, *8*(4), e62260. https://doi.org/10.1371/journal.pone.0062260

Charlton, A. J., May, C., Luikinga, S. J., Burrows, E. L., Hyun Kim, J., Lawrence, A. J., & Perry, C. J. (2019). Chronic voluntary alcohol consumption causes persistent cognitive deficits and cortical cell loss in a rodent model. *Scientific Reports*, *9*, 18651. https://doi.org/10.1038/s41598-019-55095-w

Cortez, I., Rodgers, S. P., Kosten, T. A., & Leasure, J. L. (n.d.). Sex and Age Effects on Neurobehavioral Toxicity Induced by Binge Alcohol. *Brain Plasticity*, *6*(1), 5–25. https://doi.org/10.3233/BPL-190094

Das, J. (2020). SNARE complex-associated proteins and alcohol. *Alcoholism, Clinical and Experimental Research*, *44*(1), 7–18. https://doi.org/10.1111/acer.14238

Elvig, S. K., McGinn, M. A., Smith, C., Arends, M. A., Koob, G. F., & Vendruscolo, L. F. (2021). Tolerance to Alcohol: A Critical Yet Understudied Factor in Alcohol Addiction. *Pharmacology, Biochemistry, and Behavior*, *204*, 173155. https://doi.org/10.1016/j.pbb.2021.173155

Garrett, J. E., & Wellman, C. L. (2009). Chronic stress effects on dendritic morphology in medial prefrontal cortex: Sex differences and estrogen dependence. *Neuroscience*, *162*(1), 195–207. https://doi.org/10.1016/j.neuroscience.2009.04.057

Ferguson, L. B., Roberts, A. J., Mayfield, R. D., & Messing, R. O. (2022). Blood and brain gene expression signatures of chronic intermittent ethanol consumption in mice. *PLoS Computational Biology*, *18*(2), e1009800. https://doi.org/10.1371/journal.pcbi.1009800

Flores-Bonilla, A., & Richardson, H. N. (2020). Sex Differences in the Neurobiology of Alcohol Use Disorder. *Alcohol Research : Current Reviews*, *40*(2), 04. https://doi.org/10.35946/arcr.v40.2.04

Garrett, J. E., & Wellman, C. L. (2009). Chronic stress effects on dendritic morphology in medial prefrontal cortex: Sex differences and estrogen dependence. *Neuroscience*, *162*(1), 195–207. https://doi.org/10.1016/j.neuroscience.2009.04.057

Hamson, D. K., Roes, M. M., & Galea, L. A. M. (2016). Sex Hormones and Cognition: Neuroendocrine Influences on Memory and Learning. In *Comprehensive Physiology* (pp. 1295–1337). John Wiley & Sons, Ltd. https://doi.org/10.1002/cphy.c150031

Huitron-Resendiz, S., Nadav, T., Krause, S., Cates-Gatto, C., Polis, I., & Roberts, A. J. (2018). Effects of Withdrawal from Chronic Intermittent Ethanol Exposure on Sleep Characteristics of Female and Male Mice. *Alcoholism, Clinical and Experimental Research*, *42*(3), 540–550. https://doi.org/10.1111/acer.13584

Hyer, M. M., Phillips, L. L., & Neigh, G. N. (2018). Sex Differences in Synaptic Plasticity: Hormones and Beyond. *Frontiers in Molecular Neuroscience*, *11*. https://www.frontiersin.org/articles/10.3389/fnmol.2018.00266

Jahn, R., & Scheller, R. H. (2006). SNAREs—Engines for membrane fusion. *Nature Reviews Molecular Cell Biology*, *7*(9), 631–643. https://doi.org/10.1038/nrm2002

Jobson, D. D., Hase, Y., Clarkson, A. N., & Kalaria, R. N. (2021). The role of the medial prefrontal cortex in cognition, ageing and dementia. *Brain Communications*, *3*(3), fcab125. https://doi.org/10.1093/braincomms/fcab125

Jones, S. A., Lueras, J. M., & Nagel, B. J. (2018). Effects of Binge Drinking on the Developing Brain. *Alcohol Research : Current Reviews*, *39*(1), 87–96.

Klenowski, P. M. (2018). Emerging role for the medial prefrontal cortex in alcohol-seeking behaviors. *Addictive Behaviors*, *77*, 102–106. https://doi.org/10.1016/j.addbeh.2017.09.024

Koob, G. F., & Volkow, N. D. (2016). Neurobiology of addiction: A neurocircuitry analysis. *The Lancet. Psychiatry*, *3*(8), 760–773. https://doi.org/10.1016/S2215-0366(16)00104-8

Kvamme, T. L., Schmidt, C., Strelchuk, D., Chang-Webb, Y. C., Baek, K., & Voon, V. (2016). Sexually dimorphic brain volume interaction in college-aged binge drinkers. *NeuroImage. Clinical*, *10*, 310–317. https://doi.org/10.1016/j.nicl.2015.12.004

Li, M., Cabrera-Garcia, D., Salling, M. C., Au, E., Yang, G., & Harrison, N. L. (2021). Alcohol reduces the activity of somatostatin interneurons in the mouse prefrontal cortex: A neural basis

for its disinhibitory effect? *Neuropharmacology*, *188*, 108501. https://doi.org/10.1016/j.neuropharm.2021.108501

Logrip, M. L., Oleata, C., & Roberto, M. (2017). Sex differences in responses of the basolateral-central amygdala circuit to alcohol, corticosterone and their interaction. *Neuropharmacology*, *114*, 123–134. https://doi.org/10.1016/j.neuropharm.2016.11.021

Macht, V., Elchert, N., & Crews, F. (2020). Adolescent Alcohol Exposure Produces Protracted Cognitive-Behavioral Impairments in Adult Male and Female Rats. *Brain Sciences*, *10*(11), 785. https://doi.org/10.3390/brainsci10110785

Martens, S., & McMahon, H. T. (2008). Mechanisms of membrane fusion: Disparate players and common principles. *Nature Reviews Molecular Cell Biology*, *9*(7), Article 7. https://doi.org/10.1038/nrm2417

Morningstar, M. D., Barnett, W. H., Goodlett, C. R., Kuznetsov, A., & Lapish, C. C. (2021). Understanding ethanol's acute effects on medial prefrontal cortex neural activity using state-space approaches. *Neuropharmacology*, *198*, 108780. https://doi.org/10.1016/j.neuropharm.2021.108780

Murty, V. P., Ballard, I. C., & Adcock, R. A. (2017). Hippocampus and Prefrontal Cortex Predict Distinct Timescales of Activation in the Human Ventral Tegmental Area. *Cerebral Cortex*, *27*(2), 1660–1669. https://doi.org/10.1093/cercor/bhw005

Patel, R. R., Wolfe, S. A., Bajo, M., Abeynaike, S., Pahng, A., Borgonetti, V., D'Ambrosio, S., Nikzad, R., Edwards, S., Paust, S., Roberts, A. J., & Roberto, M. (2021). IL-10 normalizes aberrant amygdala GABA transmission and reverses anxiety-like behavior and dependence-induced escalation of alcohol intake. *Progress in Neurobiology*, *199*, 101952. https://doi.org/10.1016/j.pneurobio.2020.101952

Peltier, M. R., Verplaetse, T. L., Mineur, Y. S., Petrakis, I. L., Cosgrove, K. P., Picciotto, M. R., & McKee, S. A. (2019). Sex differences in stress-related alcohol use. *Neurobiology of Stress*, *10*, 100149. https://doi.org/10.1016/j.ynstr.2019.100149

Pignataro, L., Varodayan, F. P., Tannenholz, L. E., & Harrison, N. L. (2009). The regulation of neuronal gene expression by alcohol. *Pharmacology & Therapeutics*, *124*(3), 324–335. https://doi.org/10.1016/j.pharmthera.2009.09.002

Raptis, A., Torrejón-Escribano, B., Gómez de Aranda, I., & Blasi, J. (2005). Distribution of synaptobrevin/VAMP 1 and 2 in rat brain. *Journal of Chemical Neuroanatomy*, *30*(4), 201–211. https://doi.org/10.1016/j.jchemneu.2005.08.002

Salling, M. C., Skelly, M. J., Avegno, E., Regan, S., Zeric, T., Nichols, E., & Harrison, N. L. (2018). Alcohol Consumption during Adolescence in a Mouse Model of Binge Drinking Alters the Intrinsic Excitability and Function of the Prefrontal Cortex through a Reduction in the Hyperpolarization-Activated Cation Current. *The Journal of Neuroscience*, *38*(27), 6207–6222. https://doi.org/10.1523/JNEUROSCI.0550-18.2018

Section 5 PE Tables – Results from the 2021 National Survey on Drug Use and Health: Detailed Tables, SAMHSA, CBHSQ. (n.d.). Retrieved April 18, 2023, from https://www.samhsa.gov/data/sites/default/files/reports/rpt39441/NSDUHDetailedTabs2021/NSDUHDetailedTabs2021/NSDUHDetTabsSect5pe2021.htm#tab5.6a

Seemiller, L. R., & Gould, T. J. (2020). The effects of adolescent alcohol exposure on learning and related neurobiology in humans and rodents. *Neurobiology of Learning and Memory*, *172*, 107234. https://doi.org/10.1016/j.nlm.2020.107234

Squeglia, L. M., Sorg, S. F., Schweinsburg, A. D., Wetherill, R. R., Pulido, C., & Tapert, S. F. (2012). Binge drinking differentially affects adolescent male and female brain morphometry. *Psychopharmacology*, *220*(3), 529–539. https://doi.org/10.1007/s00213-011-2500-4

Tavares, E. R., Silva-Gotay, A., Vargas Riad, W., Bengston, L., & N. Richardson, H. (2019). Sex Differences in the Effect of Alcohol Drinking on Myelinated Axons in the Anterior Cingulate Cortex of Adolescent Rats. *Brain Sciences*, *9*(7), Article 7. https://doi.org/10.3390/brainsci9070167

Varodayan, F. P., & Harrison, N. L. (2013). HSF1 transcriptional activity mediates alcohol induction of Vamp2 expression and GABA release. *Frontiers in Integrative Neuroscience*, *7*, 89. https://doi.org/10.3389/fnint.2013.00089

Varodayan, F. P., Pignataro, L., & Harrison, N. L. (2011). Alcohol Induces Synaptotagmin 1 Expression in Neurons via Activation of Heat Shock Factor 1. *Neuroscience*, *193*, 63–71. https://doi.org/10.1016/j.neuroscience.2011.07.035

Varodayan, F. P., Sidhu, H., Kreifeldt, M., Roberto, M., & Contet, C. (2018). Morphological and functional evidence of increased excitatory signaling in the prelimbic cortex during ethanol withdrawal. *Neuropharmacology*, *133*, 470–480. https://doi.org/10.1016/j.neuropharm.2018.02.014

West, R. K., Rodgers, S. P., & Leasure, J. L. (2021). Neural Perturbations Associated With Recurrent Binge Alcohol in Male and Female Rats. *Alcoholism, Clinical and Experimental Research*, *45*(2), 365–374. https://doi.org/10.1111/acer.14529

Worst, T. J., & Vrana, K. E. (2005). Alcohol and Gene Expression in the Central Nervous System. *Alcohol and Alcoholism*, *40*(1), 63–75. https://doi.org/10.1093/alcalc/agh119

Yang, Z., Gou, L., Chen, S., Li, N., Zhang, S., & Zhang, L. (2017). Membrane Fusion Involved in Neurotransmission: Glimpse from Electron Microscope and Molecular Simulation. *Frontiers in Molecular Neuroscience*, *10*. https://www.frontiersin.org/articles/10.3389/fnmol.2017.00168

Table 1: rt-PCR Forward and Reverse Primers for Housekeeper *Actinb* and Target Genes *Hsf1, Syt1, Vamp1, Vamp2.*

Gene name	Gene symbol	Forward primer	Reverse primer
Heat Shock Transcription Factor 1	*Hsf1*	GCTGATGAAGGGGAAACAGGAG	AGGAACTGAATGAGCTTGTTGAC
Synaptotagmin-1	*Syt1*	AGAGTGCTGAGAAAGAAGAGCAAG	CCGTTCTGCATCAGGTGAATC
Vesicle-Associated Membrane Protein 1 (Synaptobrevin 1)	*Vamp1*	GGTCCTCCTCCCAACATGAC	ATGTCCACCACCTCCTCCA
Vesicle-Associated Membrane Protein 2 (Synaptobrevin 2)	*Vamp2*	TGGTGGAAAAACCTCAAGATGATG	TTCTTAGGCAGGGCAGACTC
Actin beta (Beta-actin)	*Actb*	TCCAGCCTTCCTTCTTGGGTAT	GTCTTTACGGATGTCAACGTCAC

Table 2: Summary of rt-PCR one-way ANOVA statistical outcomes.

Sex	Target Gene	*F* (DFn, DFd)	*p* value	Significance
Male	*Actinb*	F (2, 30) = 1.693	p = 0.2010	ns
	Hsf1	F (2, 30) = 0.2972	p = 0.7451	ns
	Vamp1	**F (2, 29) = 10.64**	**p = 0.0003**	***
	Vamp2	F (2, 28) = 1.872	p = 0.1726	ns
	Syt1	F (2, 27) = 2.164	p = 0.1344	ns
Female	*Actinb*	F (2, 26) = 1.397	p = 0.2652	ns
	Hsf1	**F (2, 25) = 4.670**	**p = 0.0189**	*
	Vamp1	F (2, 25) = 2.285	p = 0.1226	ns
	Vamp2	**F (2, 23) = 9.174**	**p = 0.0012**	**
	Syt1	F (2, 23) = 0.04015	p = 0.9607	ns

Table 3: Summary of BEC – Gene Expression correlation statistical outcomes.

Sex	Target Gene	Pearson r	R squared	*p* value	Significance
Male	*Hsf1*	-0.0863	0.0074	$p = 0.7100$	ns
	Vamp1	-0.0579	0.0034	$p = 0.8083$	ns
	Vamp2	0.1121	0.0126	$p = 0.6378$	ns
	Syt1	0.0994	0.0099	$p = 0.6855$	ns
Female	*Hsf1*	0.0990	0.0098	$p = 0.7053$	ns
	Vamp1	-0.1868	0.0349	$p = 0.4580$	ns
	Vamp2	-0.0175	0.0003	$p = 0.9505$	ns
	Syt1	0.2799	0.0784	$p = 0.3122$	ns

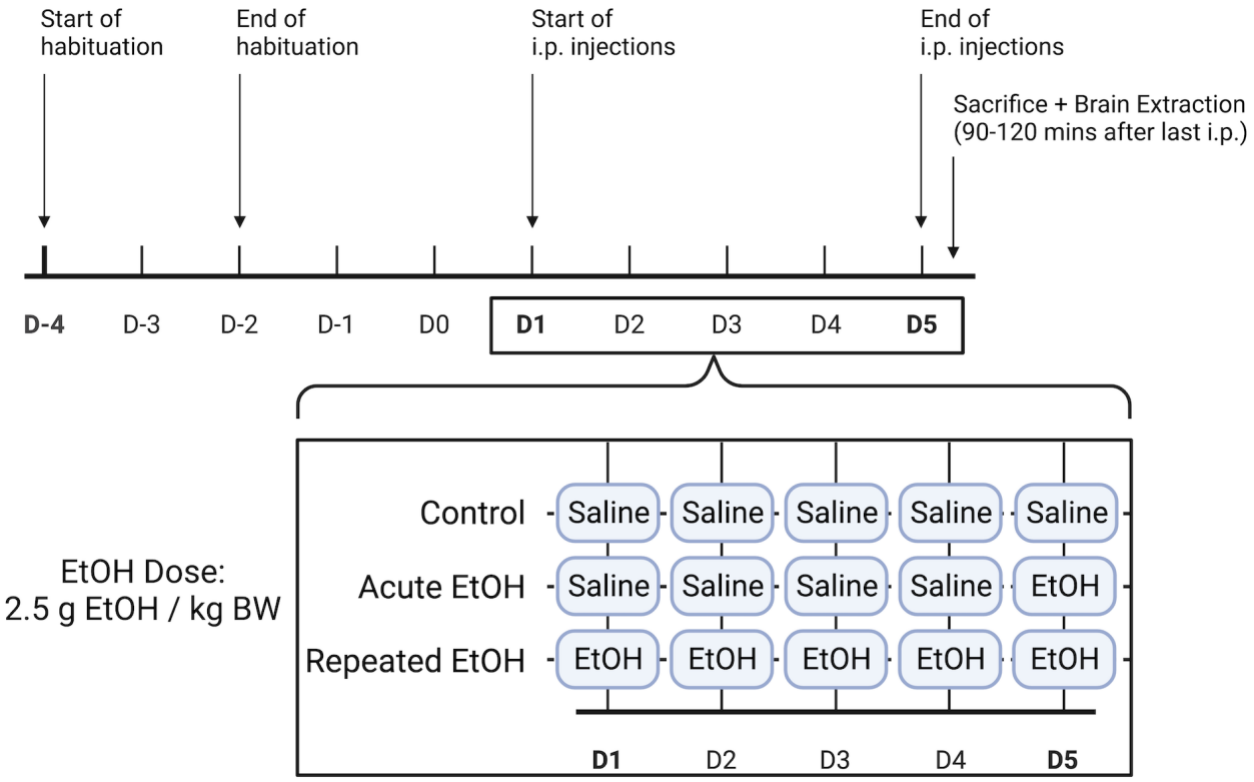

Figure 1. Treatment Timeline. Animals were randomly separated into three groups: control, acute ethanol exposure, and repeated ethanol exposure. Prior to injections, mice were habituated for four hours a day for three days, then given two days off. Control mice were intraperitoneally (i.p.) injected with physiological saline for all five days. Acute ethanol exposure mice were i.p. injected with physiological saline for the first four days and were injected with 2.5 g EtOH in saline / kg body weight on the last day. Repeated ethanol exposure mice were i.p injected with 2.5 g EtOH in saline / kg body weight for all five days. Sacrifice and brain extraction was performed 90-120 minutes after the final i.p injection for all mice (made with Biorender).

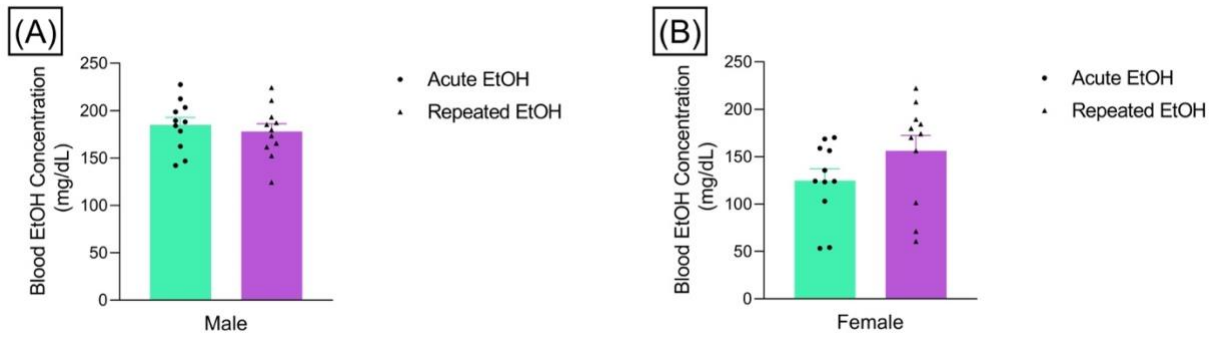

Figure 2. BECs for male and female mice do not differ between acute and repeated ethanol-treated groups. A-B: There were no significant differences in BEC between acute and repeated ethanol-treated groups for both **(A)** males and **(B)** females. N = 11 mice per group. All data are presented as mean ± SEM.

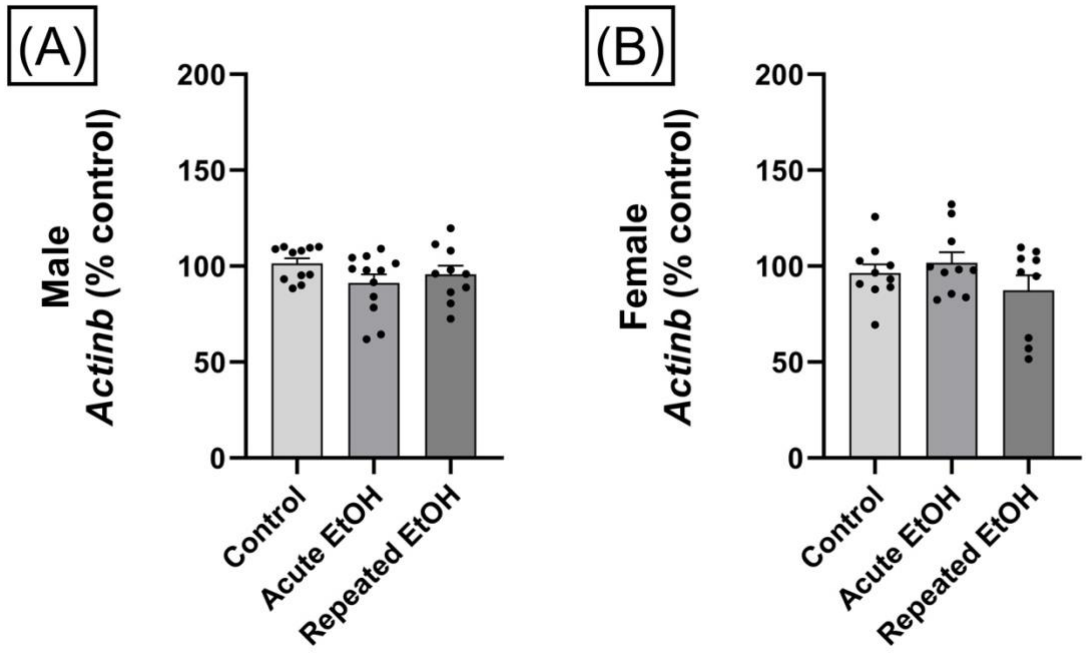

Figure 3. *Actinb* is an appropriate housekeeper gene for the male and female mPFC. A-B: *Actinb* mRNA levels were comparable between the control, acute ethanol-treated, and repeated ethanol-treated groups in the mPFC of (**A**) male and (**B**) female mice. N = 9-12 mice per group. All data are presented as mean ± SEM.

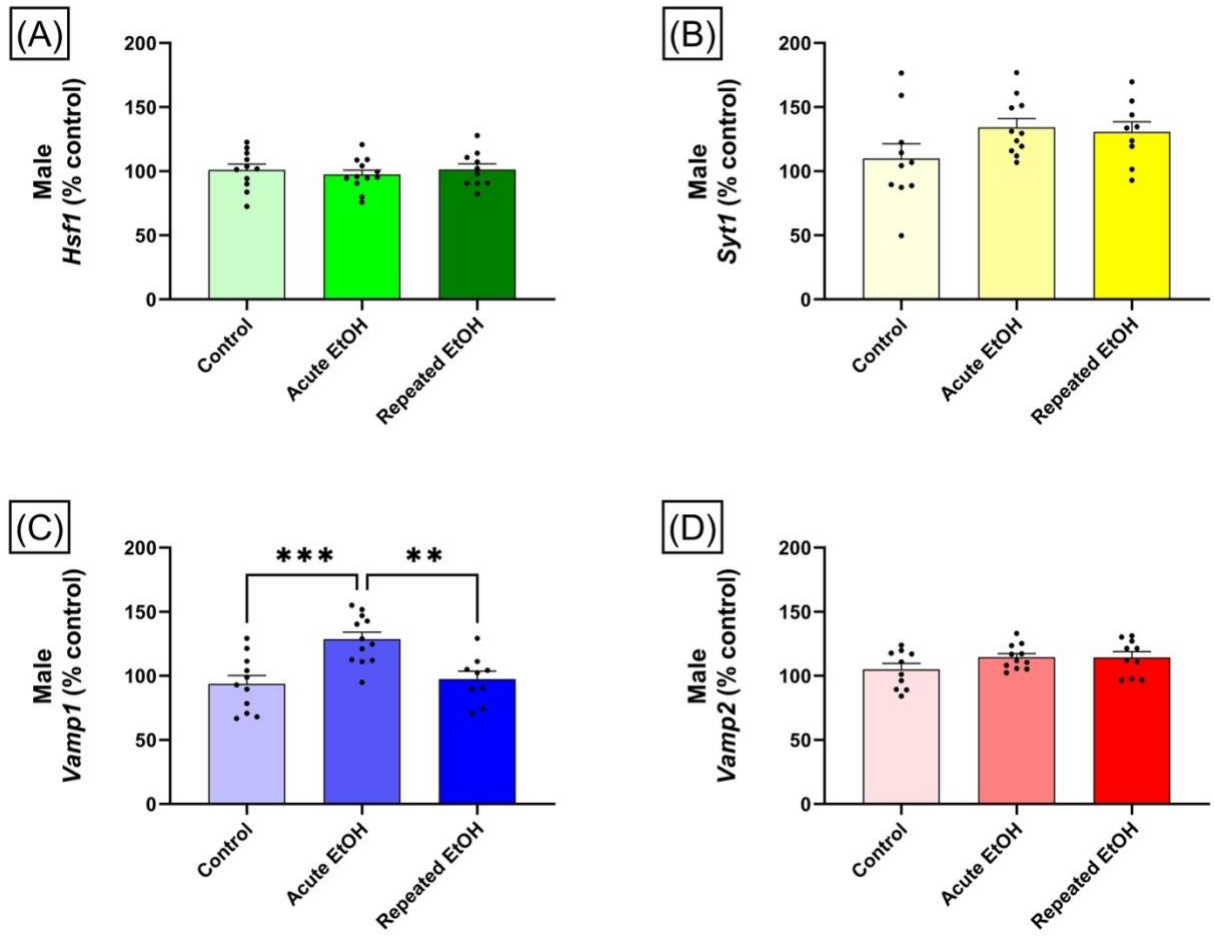

Figure 4. Acute ethanol exposure increases the gene expression of *Vamp1*, but not *Vamp2* in the male mPFC. A-B: mPFC mRNA levels in males for (**A**) *Hsf1* and (**B**) *Syt1* show no significant differences across groups. **C:** mPFC mRNA levels in males for *Vamp1* show a significant difference between the control and acute ethanol-treated groups and between the acute ethanol-treated and repeated ethanol-treated groups. **D:** *Vamp2* mRNA levels in the mPFC of males showed no significant differences across groups. N = 9-12 mice per group. All data are presented as mean ± SEM. $^{**}p < 0.01$ and $^{***}p < 0.001$ by one-way ANOVA and *post hoc* Tukey's multiple comparisons test.

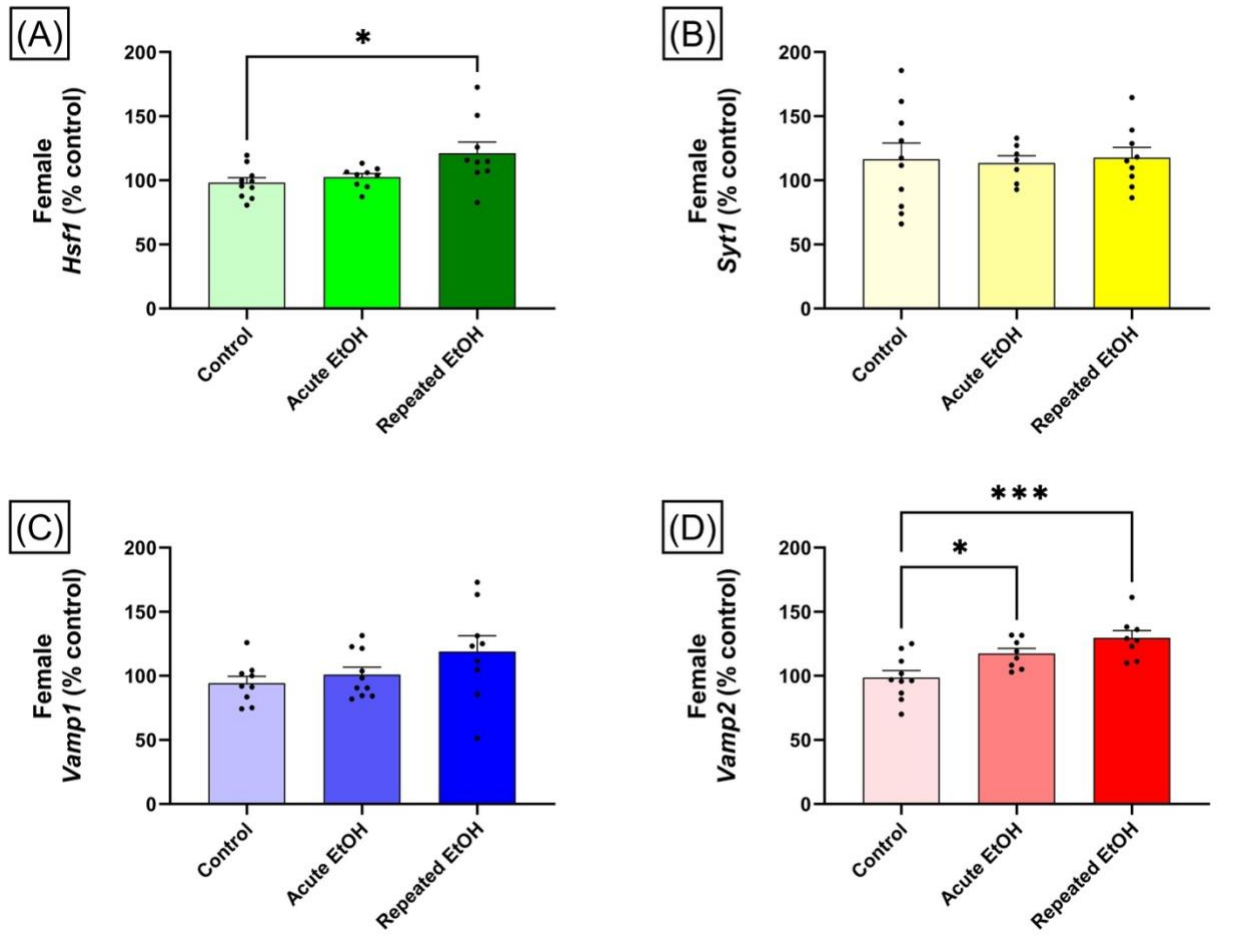

Figure 5. Repeated ethanol exposure increases the gene expression of *Hsf1* and acute and repeated ethanol exposure both increase the gene expression of *Vamp2*, but not *Vamp1* in the female mPFC. A: mPFC mRNA levels in females for *Hsf1* show significant differences between the control and repeated ethanol-treated groups. **B-C:** mPFC mRNA levels in females for (**B**) *Syt1* and (**C**) *Vamp1* show no significant differences across groups. **D:** mPFC mRNA levels in females for *Vamp2* show significant differences between the control and acute ethanol-treated groups and between the control and repeated ethanol-treated groups. N = 9-12 mice per group. All data are presented as mean ± SEM. *$p < 0.05$ and ***$p < 0.001$ by one-way ANOVA and *post hoc* Tukey's multiple comparisons test.

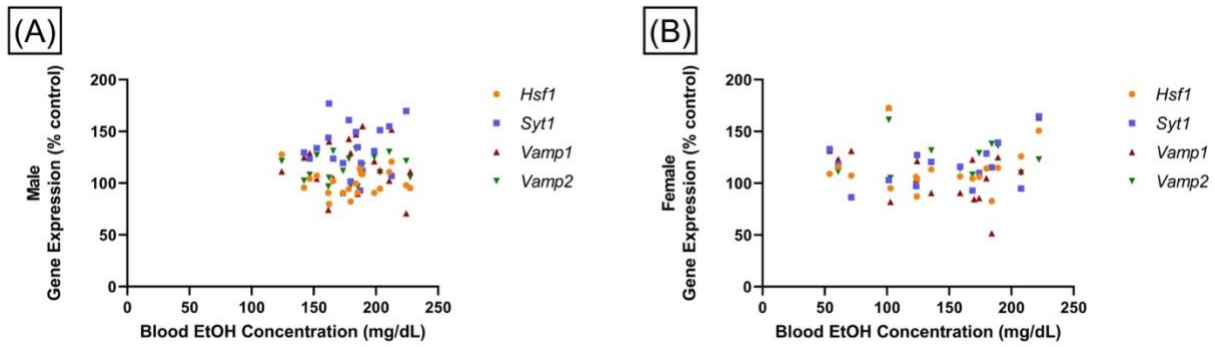

Figure 6. BEC was not significantly correlated with normalized gene expression for ethanol-treated males or females. A-B: mPFC mRNA levels of *Hsf1*, *Vamp1*, *Vamp2*, and *Syt1* normalized to controls were not significantly correlated with BEC in **(A)** male or **(B)** female mice. N = 11 mice per group. All data are presented as mean ± SEM.