ABSTRACT

Intermittent Ethanol-induced Fear Memory Impairment with Differential Hippocampal Activation in Male Mice

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The limbic system, particularly the hippocampus and amygdala, are oft-studied and well-established regions for fear learning and memory and have been seen to critically underlie the encoding of contextual fear memory. Literature has shown that chronic ethanol consumption has a negative effect on hippocampal beta-dynorphin – a neuropeptide implicated in limbic LTP and fear-forming memories. The impact of self-administered chronic ethanol on hippocampal fear memory has not yet been investigated in mice. In this study, we exposed 3 cohorts of C57BL/6J mice to an intermittent-access ethanol protocol for 6 weeks followed by fear conditioning tests. We found significant differences in freezing behaviour between binge-like drinking mice and water controls on a trace conditioning protocol. Immunohistochemistry for c-Fos was performed, and significant differences were seen in CA1, CA3, and the dentate gyrus between binge-like mice and controls after fear exposure. These findings suggest that chronic ethanol consumption has a dampening effect on fear learning in mice and implicates differential hippocampal activation potentially underlying the behaviour seen.

APPROVED BY DIRECTOR OF HONORS THESIS:

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INTERMITTENT ETHANOL-INDUCED FEAR MEMORY IMPAIRMENTS WITH DIFFERENTIAL HIPPOCAMPAL ACTIVATION IN MALE MICE

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CHAPTER ONE

Introduction

The scourge of alcohol-use disorder (AUD) and binge-drinking abuse wrest a costly toll on society. According to a recent nationwide survey on drug use and health conducted by the National Institute of Alcohol Abuse and Alcoholism, more than 29.5 million individuals in the United States alone suffer from alcohol abuse (SAMHSA, Centre for Behavioural Health Statistics and Quality, 2021). More shockingly, 32 people in the United States lose their lives every day due to incidents involving drunk-driving - a statistic that equates to one death every 45 minutes (NHTSA, 2022). Such preventable loss of life is utterly unacceptable and, in accord with this study and the rest of the addiction field, ought to be remediated. In recent years, advances in neuroscientific techniques have provided new insights into the neural circuits and molecular mechanisms that regulate behaviour, including those involved in chronic alcohol consumption and addiction. Animal models have been particularly valuable in this regard, allowing researchers to investigate the effects of ethanol on behaviour and neural function with high precision and control. The human emotion of fear is a multifaceted biological process that involves subjective emotional experiences and its interplay with physiological-behavioural reactions to frightful stimuli. Outside of our experience with fear emotion, an organism's ability for threat detection and behavioural adaption can be said to be a natural part of the fear response and can be used to quantify non-human fear learning (Raber et al., 2019). Fear learning is a critical cognitive process that allows animals to respond appropriately to potential threats in the environment and increase survivability should similar threats arrive

in the future. Impairments in fear learning has been consistently observed in a range of psychiatric disorders, especially anxiety disorders and post-traumatic stress disorder (Holmes et al., 2012). Given the high comorbidity between alcoholism and these neuropsychiatric disorders, understanding the neural mechanisms underlying ethanol-induced changes in fear learning may have important implications for the treatment of both conditions. Specifically, elucidating the molecular and cellular changes that occur following ethanol exposure may help identify novel targets for pharmacological intervention in the treatment of AUDs and associated disorders. As yet one more pivotal étude in the collective concerto of research into alcohol addiction, our study strives to lay a stronger foundation for future translational research that explores how alcohol-use disorder affects human decision-making in the face of fear-related events or precipitates reckless behaviour. The rationale behind of our study is to expand our comprehension of how ethanol, particularly in the context of binge-like drinking, may impede the natural fear response in mice.

According to the Centre for Disease Control's (CDC), for each year in the United States, 140,000 deaths and over $249 billion in economic costs are products of excessive alcohol use, shortening lives by an average of 26 years (CDC, 2022). The CDC defines binge drinking as "consuming four or more drinks on an occasion for women or five or more drinks on an occasion for a man" (CDC, 2012). Chronic health effects of AUD include higher susceptibility to cardiovascular diseases, liver disease, cancer, and long-term cognitive impairments.

The American Psychiatric Association's Diagnostic and Statistics Manual-IV, Text Revision (DSM-IV-TR) currently provides these standardized criteria for diagnosing

alcoholism:

A. A maladaptive pattern of substance use leading to clinically significant impairment or distress, as manifested by two or more of the following, occurring at any time in the same 12-month period:

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 and/or strong desire to use alcohol.

5. Recurrent alcohol use resulting in a failure to fulfil 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. Tolerance, as defined by either of the following:

- need for markedly increased amounts of alcohol to achieve intoxication.
- a markedly diminished effect with continued use of the same amount of alcohol.

To study the effects of chronically elevated alcohol intake, also called binge-like drinking, on fear behaviour, a paradigm ought to be utilized that can induce animals to voluntarily drink high amounts of alcohol. Intermittent access (IA) to drugs of abuse, 20% ethanol in this case, can be defined as an interval presentation of free-choice ethanol to

subjects, contrasting a continuous access schedule where subjects would have unremitting access to drink. Intermittent 24-hour cycles of ethanol access and deprivation can generate high levels of voluntary ethanol drinking in C57BL/6J male mice (Hwa et al., 2011). IA seems to be a predominant feature of many conditions that display high amounts of alcohol drinking (Falk and Tang, 1988; Rodd-Henrick et al., 2000). This type of drinking schedule has been shown to induce a propellant transition from moderate to escalated drug use where repeated cycles of binge lead to larger compensatory relapse due to accentuated withdrawal symptoms (Ballenger and Post, 1978; Becker, 1998). Ultimately, an intermittent access protocol is a valuable and translatable tool for approximating human alcoholic-like drinking. This study utilized the IA protocol to induce high voluntary EtOH consumption in mice given free choice of 20% EtOH to engender a reliable model of binge drinking behaviour.

As mentioned earlier, threat detection and fear learning therein are paramount to organism survivability. When one encounters stress, the formation of fear memories can prove a fortuitous occurrence, enabling individuals to steer clear of similar circumstances and hone future coping abilities. Fear learning is a swift and puissant process, for even a sole exposure to potent stress can engender fear memories of considerable longevity. Yet, adaptation to a fluctuating environment also necessitates the extinction of these memories. Extinction learning, more intricate than fear learning, frequently mandates numerous nonreinforced exposures to fear-associated cues or context and proceeds at a slower pace (Bilkei-Gorzo et al., 2012). While fear learning essentially resembles classical Pavlovian conditioning, extinction learning is a multifarious process requiring the recall of the fear

memory and detection of the prediction error to establish and cement a new safety memory.

It is well established that the limbic system, comprising the amygdala, prefrontal cortex, and hippocampus, is a paramount brain region grounding fear memory processing. Concerning the neurochemical façon of this study, strong correlations have been seen between hippocampal beta-dynorphin activation and retarded spatial learning (Sandin et al., 1998). Beta-dynorphins are a class of opioid peptide that bind to kappa-opioid receptors (KORs) in the brain. KORs are crucial in the limbic system – brain and spinal cord areas heavily associated with memory, learning, stress, and emotional control. Additionally, long-term potentiation –the persistent strengthening of neural connections undergirding long-term memory – is modulated by dynorphins in the hippocampus. Dynorphinergic neurons hold a position of great concentration within the mossy fibre projection from the hippocampus' dentate gyrus to its CA3 region. Several lines of evidence suggest that dynorphin signalling also contributes to the formation and extinction of aversive memories in that dynorphins have been correlated to stress-induced learning and memory deficits (Carey et al., 2009). Additionally, the immediate early gene c-Fos can be employed to visualize neural activity. An immediate early gene is one that shows rapid and transiet increases in its expression to extracellular signals. Because c-Fos displays such rapid expression in response to neurotransmitter release, it is an excellent albeit wide-spread and unspecific marker for neuronal activation.

Increases in beta-dynorphin output modify long-term potentiation in a way that causes presynaptic signal inhibition due to a downregulation of glutamatergic release. The effect of chronic, longitudinal alcohol consumption on brain neurochemistry has similarly

exhibited deficits in hippocampal dependent-plasticity. Together, these prior observations lead to the hypothesis that chronic, high level of ethanol consumption impairs extinction by disrupting normative hippocampal as a result of increased hippocampal activation with the potential of beta-dynorphin implicitness. By examining the impact of binge ethanol exposure on fear learning, we aim to provide and add to the literature a more comprehensive understanding of the behavioural and neural mechanisms underlying alcohol use disorders and their associated cognitive impairments.

CHAPTER TWO

Materials and Methods

Mice

Adult C57B/6J male mice were used in this study and were procured from Jackson Laboratories (ME). They were housed in the Baylor University vivarium at an ambient temperature of 22º C, with a 12-hour light (19H00 to 07H00) and 12-hour dark reverse diurnal cycle. The raison d'être behind the use of C57BL/6Js is due to their strain's consistently elevated levels of ethanol consumption in comparison to other genetically-inbred or wildtype strains used in preclinical research (Lê, 1994; Belknap et al., 1993). This facet of C57s ensures a high and consistent level of alcohol intake across cohorts and is more translatable as a model of binge drinking. The mice were group-housed in pairs of two prior to the start of ethanol exposure and left to acclimate to vivarium conditions for approximately a week. Post-acclimation, the mice were singled housed and given three days to habituate to their new environment, as well as to a two-bottle choice paradigm whereby they have volitional access to two bottles of liquid. Following single house habituation, experimental mice were given one bottle of water and one bottle of 20% ethanol (EtOH) with controls having two bottles of water. All mice were provided with ad libitum access to food and water. Three cohorts of mice (n = 48; 24 = EtOH, 24 = H_2O) were used in the entirety of the study with a single cohort consisting of 16 mice of which half were designated as ethanol accessible and half as water controls. All procedures done to the mice followed the National Institutes of Health Guidelines for the Care and Use of

Laboratory Animals and the animal protocol was approved by Baylor University Animal Care and Use Committee.

Intermittent Alcohol Procedure

The present study utilized an intermittent alcohol protocol to institute escalated alcohol drinking behaviour. Ethanol solutions were prepared from tap water and 95% ethyl alcohol (Pharmaco-AAPER, Brookfield, CT). EtOH was presented to alcohol access-grouped mice every Monday, Wednesday, and Friday with Sunday and the days in between the MWF cycle being water-only exposure. Bottles were weighed to the nearest hundredth of a gram 24 hours after the fluids were given. To control for spillage à cause de experimenter handling or evaporation, weekly averages of loss of fluid in a cage sans animal presence – known as drip – were subtracted from individual fluid intakes. Mice were weighed to the nearest tenth of a gram before every fortnight to calculate the grams of ethanol intake per kilogram of body weight throughout their 18-week binge cycle.

Fear Conditioning & Learning

To condition fear in mice as well as perform associated fear memory tests, two fear conditioning apparatus were utilized. The apparatus (26 cm x 22 cm x 18 cm) consists of two sides that are acrylic, two sides that are metal, and a grid floor bottom which is used to deliver a mild, aversive foot shock. The testing chamber is housed in a sound attenuated chamber. The chamber is also light tight to prevent exterior illumination from influencing the apparatus' cameras and their motion detection software. The background noise in this chamber is 65 dB. The tone presented to the mice during conditioning and testing will be a

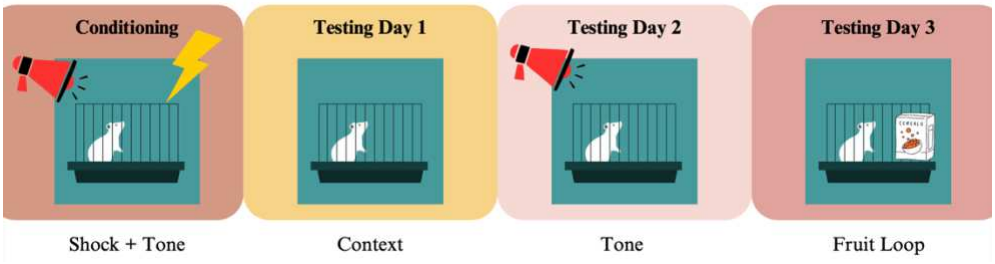
2,700 Hz tone. The foot shock was calibrated to the experimental standard of 0.5 mA, which is a low to moderate intensity according to neuroscientific criterion.

The fear protocol consisted of one conditioning day whereby both groups of mice, alcohol-access group and H_2O controls, were exposed to a 0.5mA foot shock and 2,700Hz tone every 30 seconds for 5 seconds over a total duration of 5 minutes. Following conditioning, both groups were exposed to 3 consecutive, alternating days of fear learning tests. Testing day 1 was context-based à la the mice's freezing behaviour was measured in lieu of a new context, as the hippocampus has widely been studied to be essential for contextual fear conditioning (Gewirtz, 2000). The fear chambers were newly fitted with marble inserts and a vanilla scent was sprayed on the floors of the chambers so as to provide novel contextuality to the mice. The mice were exposed to a 5-minute duration in the novel fear chamber setting, and thusly assessed for freezing behaviour between groups. Testing day 2 had the mice in the fear chamber with the same paradigm as conditioning, with the chambers being reinstated to their conditioning-day appearance, but with the removal of the foot shock. The tone was played every 30 seconds for a 5 second duration over the course of the 5 minutes. The mice's freezing behaviour was recorded throughout the course of the experiment. Testing day 3 measured freezing behaviour in context of the normative fear chamber with the addition of an appetitive substance. Rodents have high preferences for sugar-containing substances, as they are not a part of normal rodent diet, and as such find sweetened stimuli particularly motivating. To explore the relationship between binge-like behaviour and fear memory considering a high motivator, we exposed mice to a fruit loop during their being in the aversive context of the fear chamber. Prior to the third testing day, both groups were habituated to the appearance and presence of a fruit

loop which was placed in their individual home cages for consumption. During testing, a singular fruit loop was placed in the centre of the fear chamber and mice were assessed on their freezing behaviour.

Fear conditioning and subsequent testing days were split into four consecutive and alternating days over the course of a week. As an example: should fear conditioning occur on a Tuesday, testing day 1 would fall on Thursday, testing day 2 on Saturday, and so forth. Conditioning and testing occurred on ethanol-withdrawn days on the IA protocol to reduce extraneous effects such as alcohol-induced disinhibition, and to better qualify the effect of acute withdrawal from chronic drinking on fear learning. During conditioning and testing, mice not in the fear chambers were maintained in their home cage in a separate, sound-resistant room so as to not confound conditioning via second-hand habituation or sensitization of experimental stimuli.

Figure 1A: Fear Conditioning and Extinction Tests Protocol



Cohort One

The first and preliminary cohort of mice (n = 16) were split into IA and H_2O-control groups and underwent their respective drinking procedures for a 6-week period. Following the 6-week binge, both groups of mice underwent classical/delayed fear conditioning whereby the unconditioned stimulus (the foot shock) and conditioned

stimulus (tone) are not separated by an interstimulus interval. Video capture and freezing behaviour were recorded and assessed by FreezeFrame 4 software (Harvard Apparatus).

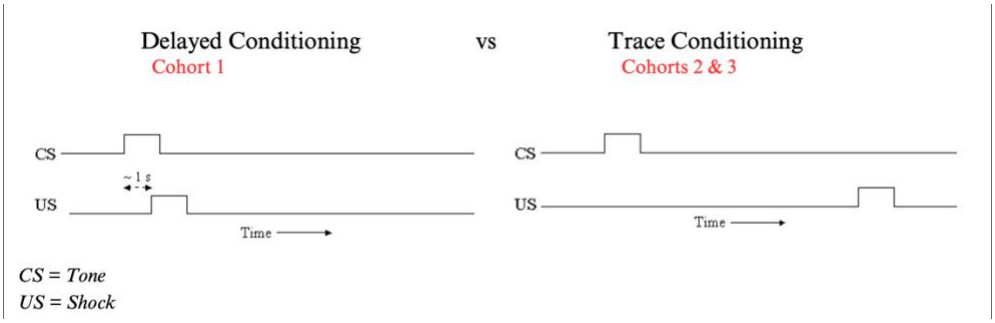
Cohort Two

In contrast to the first cohort, the second (n = 16) and third (n = 16) cohorts of mice underwent trace conditioning, a form of classical conditioning in which the presentation of the conditioned stimulus and unconditioned stimulus is separated by an interstimulus interval. This conditioning paradigm has been shown in literature to require an intact hippocampus whereas delayed/classical conditioning typically does not (Debra et al., 2006; Grover et al., 2022). The reasoning to switch from delayed conditioning of tone and shock, as seen in the first cohort, to trace was to compare learning differences between a paradigm that did and did not require normative hippocampal functioning. After mice in this cohort completed 6 weeks of drinking, they were exposed to the same protocol as mice from cohort 1 albeit with a trace period of 20s implemented between tone and shock this time.

Cohort Three

The third cohort proceeded through the same protocol as cohort 2 except with a second fear conditioning reinstatement. This secondary conditioning followed extinction tests on the subsequent non-IA day along with tissue perfusion and brain extraction in a 20-minute post-conditioning manner. The reasoning behind this extra post-extinction conditioning and time-locked tissue acquisition was in order to corroborate immunohistochemical findings to a state of fear reinstatement.

Figure 1B: Delayed vs Trace Conditioning



Immunohistochemistry & Microscopy

Because the hippocampus, particularly the dentate gyrus, CA1, and CA3, are known for their involvement in contextual and conditioned fear memory, a c-Fos immunohistochemistry assay was utilized on the third cohort of mice. Previous studies examining fear learning have shown decreases in activation of these hippocampal sites in groups where fear learning was extinguished (Knapska, 2009; Silva et al., 2019), by means of the early active gene c-Fos as a marker for active neuronal populations. Following the second round of fear conditioning in the third cohort of mice, all were perfused using the fixative paraformaldehyde (PFA) in a time-dependent manner of 20 minutes post-conditioning. Perfused brains were then sliced at 45 µm into coronal sections on a vibratome (Leica VT1200 S, Freiburg, DE) and subsequently stored in a phosphate buffered saline (PBS) solution. Brains were then stained for c-Fos immunofluorescence to visualize the localization of c-Fos expressing cells in the hippocampus to determine potential differences in hippocampal activation following a reinstatement of the aversive, fear environment after the extinction tests. All terminal procedures were performed in

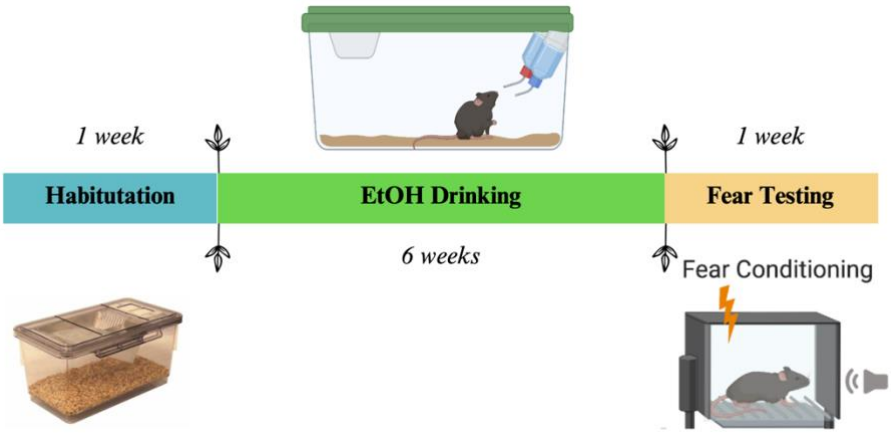
guidance with and under the approval of the Baylor University Animal Care and Use Committee.

To image c-Fos immunofluorescence in sliced mice hippocampi, a fluorescence microscope IX-81 (Olympus Corp., Shinjuku, JP) was utilized. A TRITC/Cy3-filter (ext. 530-560nm, em. 590-650 nm) and Cell Sens Dimension 2 software were used to capture images at a 10x objective lens and count c-Fos puncta (small, distinct points that can be quantified as sites of neuronal activation). c-Fos puncta were counted and paired to individual mice within their respective group in a double-blind manner.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism 9 (GraphPad Software, Inc., San Diego, CA). Regarding the drinking procedure for both the IA group and water controls, Ethanol intake (g/kg), body weight (g), volume of ethanol intake (ml), water intake (ml), total fluid intake (ml), and ethanol preference (%) during the 6-week binge period were measured and utilized in a non-linear regression (one phase decay) statistical test with days of drinking and EtOH as factors. Regarding fear conditioning and freezing behaviour quantifiability, mice freezing behaviour was operationally defined as the absence of any movement except for respiratory-related movements. Freezing behaviour was measured digitally via FreezeFrame 4 and a digital camera sensor. Differences in freezing behaviour were analysed by unpaired, parametric t-tests (two-tailed) with EtOH group and H_2O group as factors. c-Fos localization was analysed by unpaired, parametric t-tests (two-tailed) with EtOH averaged puncta count and H_20 averaged puncta count as factors.

Figure 2: Experimental Timeline



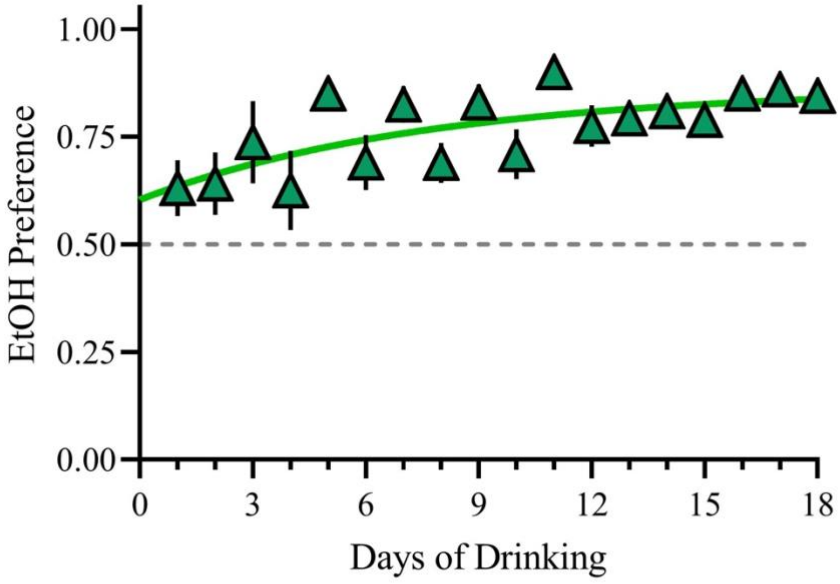
CHAPTER THREE

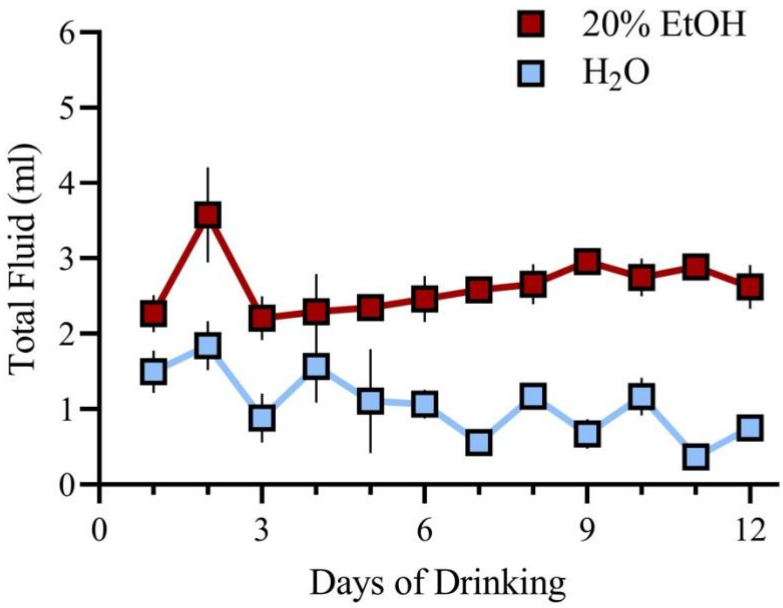
Results

IA & Drinking

In the non-linear regression analysis of the first cohort, the average of IA mice's alcohol consumption (n = 8) over 12 days of drinking were used to predict the mice's preference for ethanol on a 0 to 1 scale with 1 representing 100% EtOH preference. This IA exposure explained high EtOH preference over the course of the 12 days ($R^2 = .49$). Total fluid consumed by the IA group, measured in mL, was also collected over the timeline, and significantly higher consumption of EtOH over H2O was seen, providing basis for their preference results.

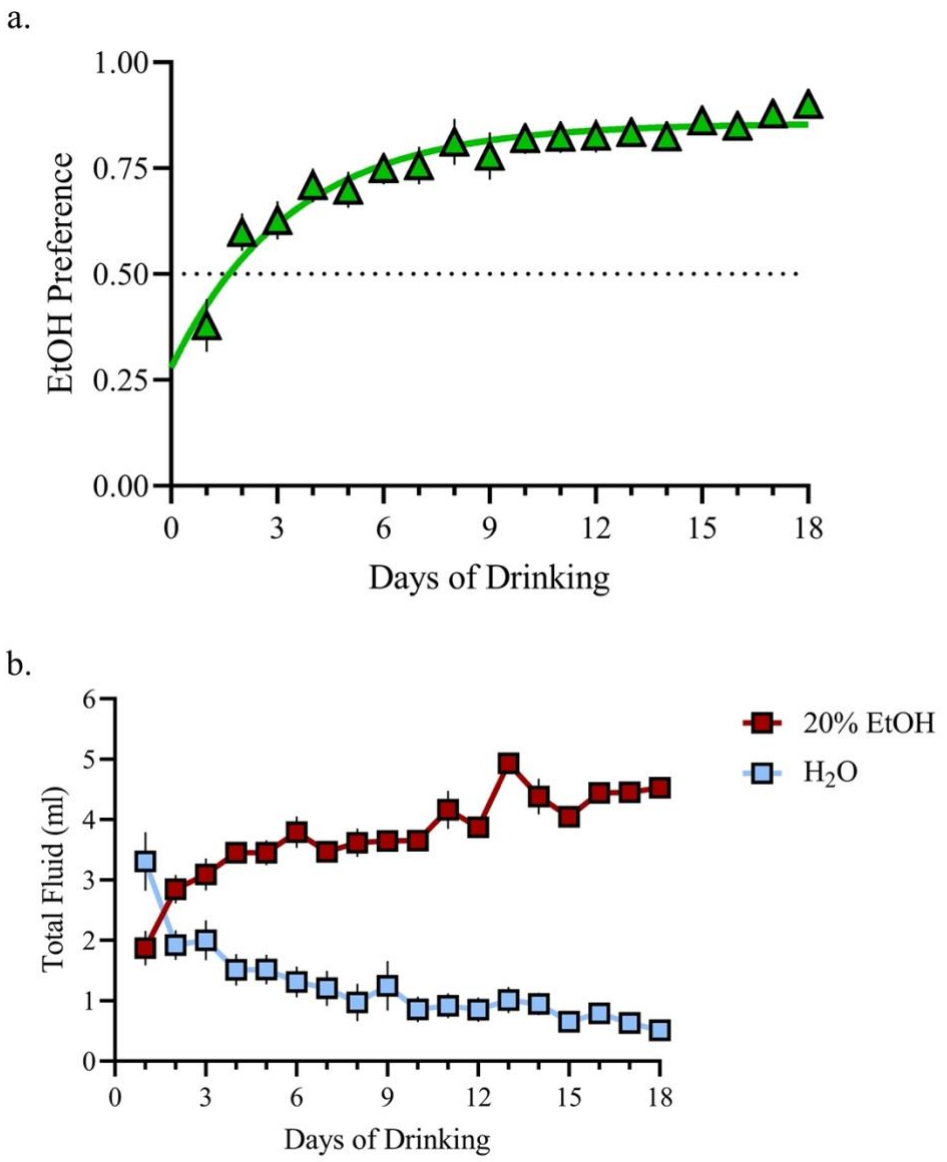
Figure 3A: Cohort One's Drinking





In the second cohort's non-linear regression analysis, the average of IA mice's alcohol consumption (n = 8) over 18 days of drinking were used to predict the mice's preference for ethanol on a 0 to 1 scale with 1 representing 100% EtOH preference. This IA exposure explained increased EtOH preference over the course of the 12 days (R^2 = .54). Total fluid consumed by the IA group, measured in mL, was also collected over the timeline, and significantly higher consumption of EtOH over H2O was seen, providing basis for their preference results.

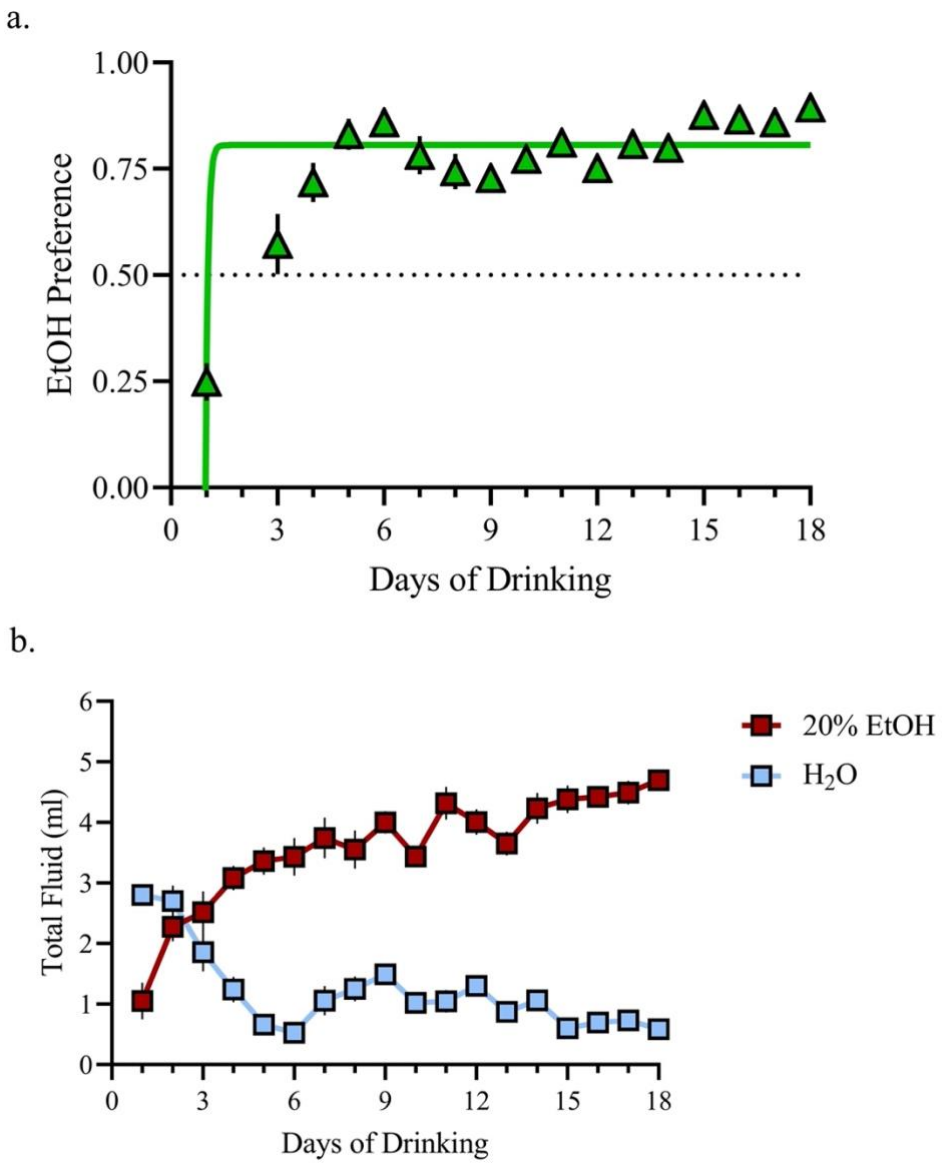
Figure 3B: Cohort Two's Drinking



In the third and final cohort's non-linear regression analysis, the average of IA mice's alcohol consumption (n = 8) over 18 days of drinking were used to predict the mice's preference for ethanol on a 0 to 1 scale with 1 representing 100% EtOH preference. This IA exposure explained increased EtOH preference over the course of the 12 days (R^2 = .49). Total fluid consumed by the IA group, measured in mL, was also collected over the

timeline, and significantly higher consumption of EtOH over H2O was seen, providing basis for their preference results.

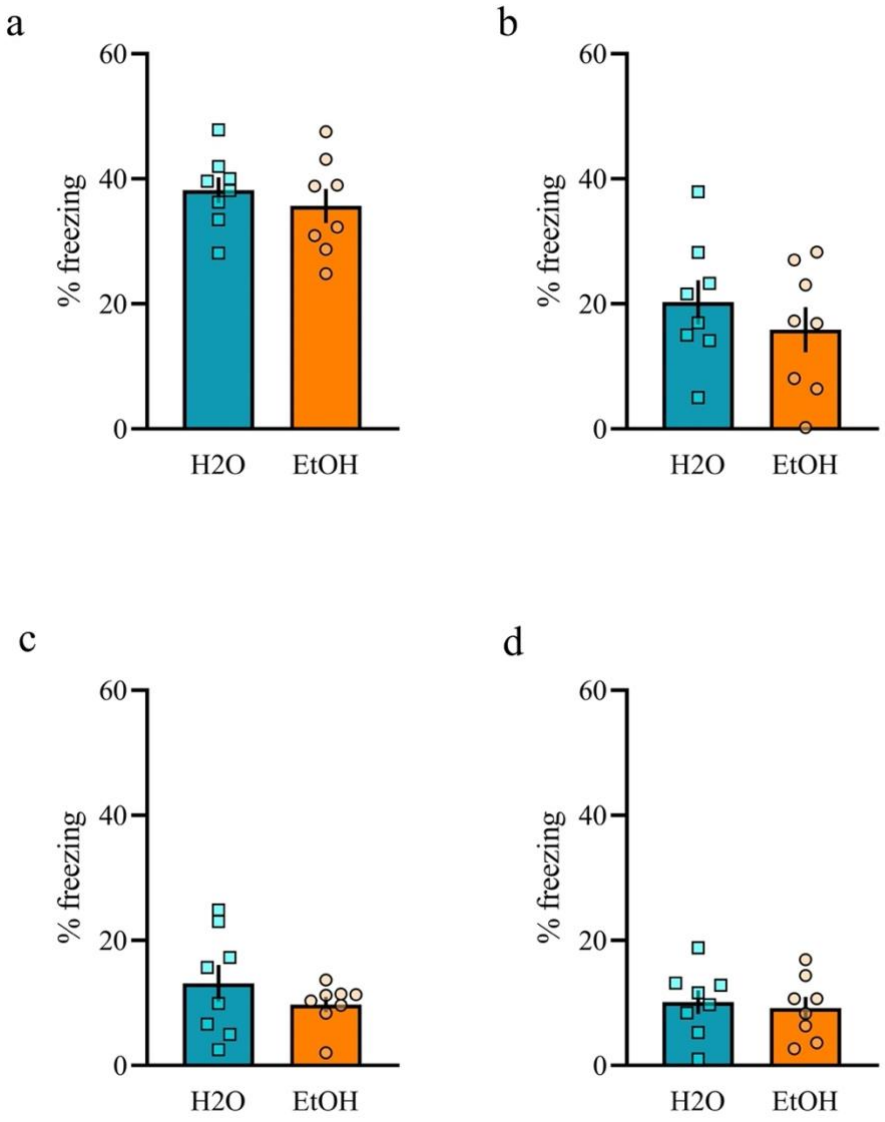
Figure 3C: Cohort Three's Drinking



Cohort One Fear Results

In these parametric two-tailed analyses of delayed fear conditioning and the three extinction tests – to contextual stimulus of the environment, the conditioned tone, and in response to an appetitive substance –no significant effects were seen between the IA group and water controls. Because a delayed classical conditioning protocol was put into effect to conditioning this cohort of mice, thereby not requiring hippocampal use, the non-significant results are coherent with the literature .

Figure 4: Cohort 1 Fear (a. delayed conditioning, b. test 1, c. test 2, d. test 3)

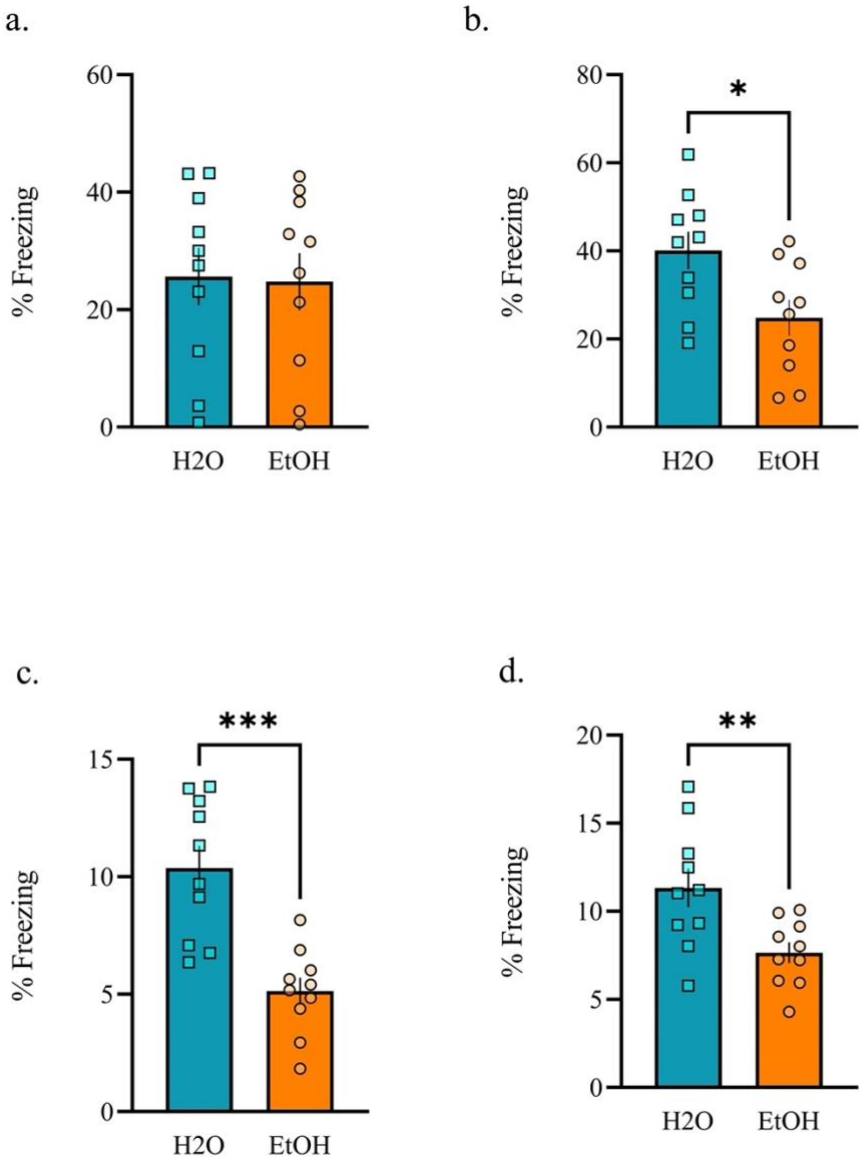


Cohort Two Fear Results

The second cohort, as expected, saw stark differences in behavioural output between experimentally assigned groups compared to the first cohort on delayed conditioning. No effect was seen between IA and water control mice during trace fear conditioning. In the first extinction test where the environment of the fear chamber served as the contextual stimulus, there was a significant difference between IA mice and water

controls [t (18) = 2.585, p = .0187] with IA mice experiencing less freezing behaviour (M = 24.84, SD = 12.88) than water controls (M = 40.08 % freezing, SD = 13.48 % freezing). The second extinction test where tone served as the CS saw significant differences between IA mice and water controls [t (18) = 4.768, p < .001]. IA mice experienced less freezing behaviour (M = 5.12 % freezing, SD = 1.81 % freezing) than water controls (M = 10.37 % freezing, SD = 2.97 % freezing). The third extinction test where the fruit loop was used as a measure disinhibition in lieu of fear memory furthermore saw significant differences between groups [t (18) = 2.950, p < .01]. IA mice experienced less freezing behaviour (M = 7.65 % freezing, SD = 1.86 % freezing) than water controls (M = 11.32 % freezing, SD = 3.48 % freezing).

Figure 5: Cohort 2 Fear (a. trace conditioning, b. test 1, c. test 2, d. test 3)



Cohort Three Fear Results

The third cohort, which was also on the trace protocol, saw similar group effects in behavioural output as the second cohort. No effect was seen between IA and water control mice during trace fear conditioning. The first extinction test yielded significant differences between IA mice and water controls [t (18) = 4.176, p < .001] with IA mice experiencing

less freezing behaviour (M = 18.48 % freezing, SD = 9.86 % freezing) than water controls (M = 36.57 % freezing, SD = 9.51 % freezing). The second extinction test saw also significant differences between IA mice and water controls [t (18) = 5.315, $p < .001$]. IA mice experienced less freezing behaviour (M = 2.27 % freezing, SD = 1.49 % freezing) than water controls (M = 9.21 % freezing, SD = 3.85 % freezing). The third extinction test again saw significant differences between groups [t (18) = 4.248, $p < .001$]. IA mice experienced less freezing behaviour (M = 3.87 % freezing, SD = 1.96 % freezing) than water controls (M = 11.15 % freezing, SD = 5.05 % freezing).

Figure 6A: Cohort 3 Extinction (a. test 1; b. test 2; c. test 3)

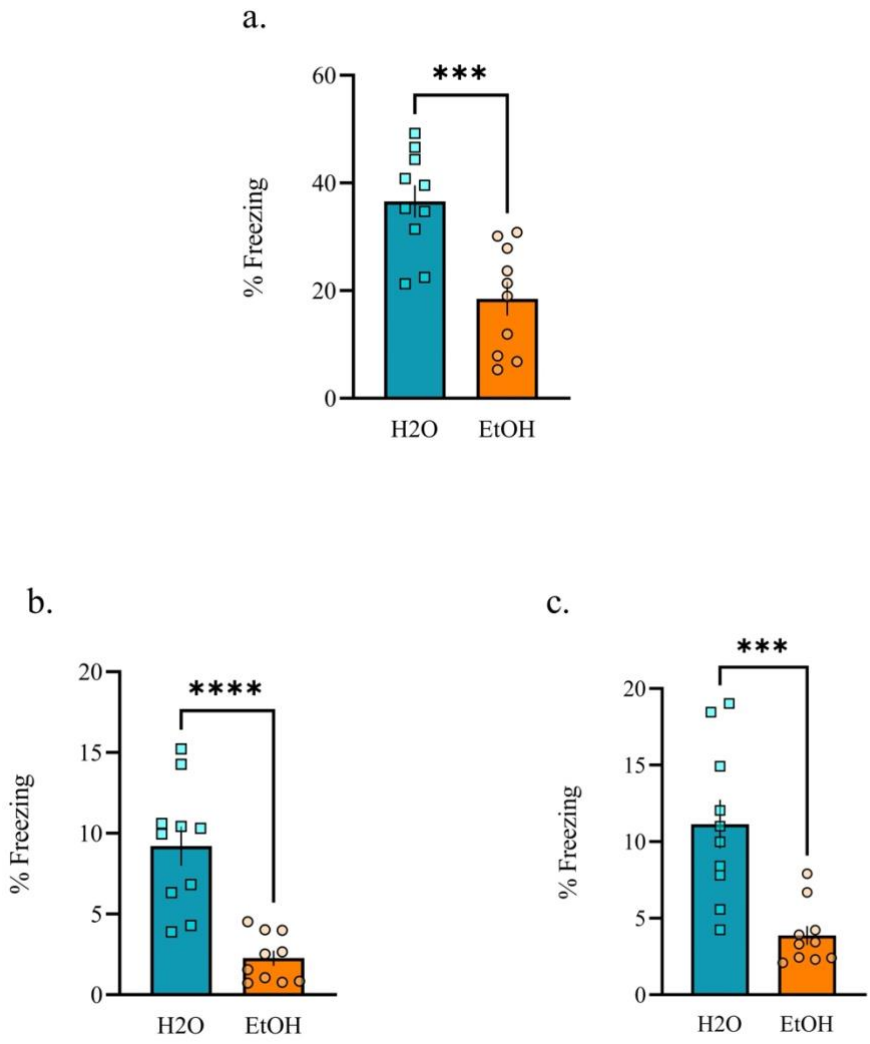
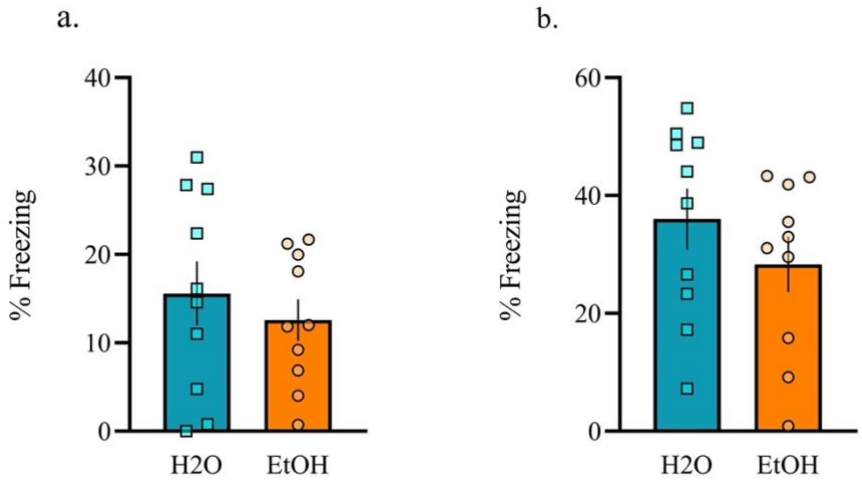


Figure 6B: Cohort 3 Conditioning (a. trace conditioning; b. trace reinstatement)



Immunohistochemistry

In the parametric two-tailed analyses of c-Fos puncta count post-reinstatement conditioning for cohort 3, a significant effect was seen between IA mice and water controls [t (14) = 7.525, p = .0001]. IA mice expressed higher puncta counts in the CA1, CA3, and dentate gyrus of the hippocampus (M = 237.0, SD = 73.23) compared to water controls (M = 37.75, SD = 15.68).

Figure 7: c-Fos Puncta Count between Groups

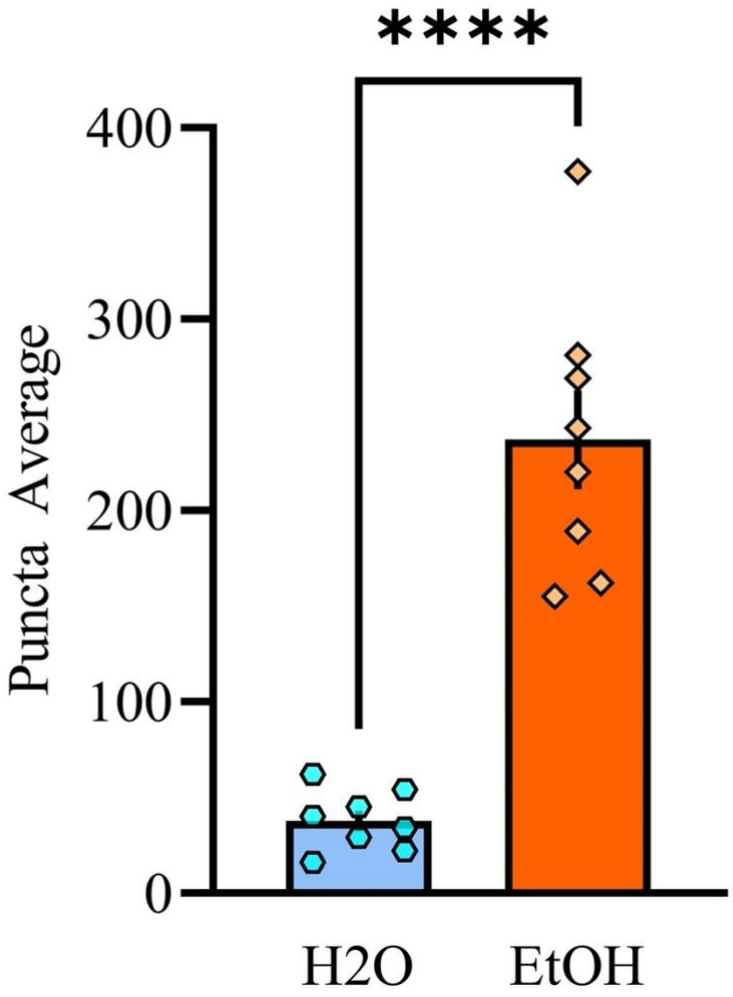


Figure 8A: IA Left Hippocampus Immunofluorescence

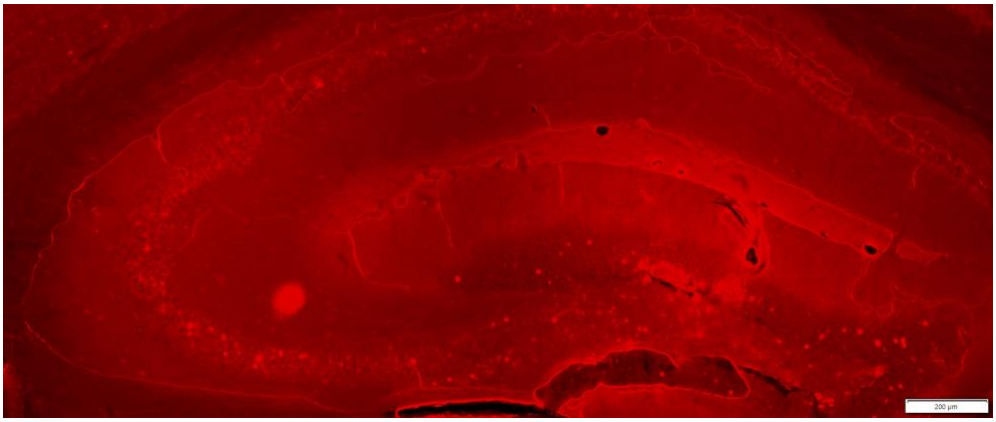
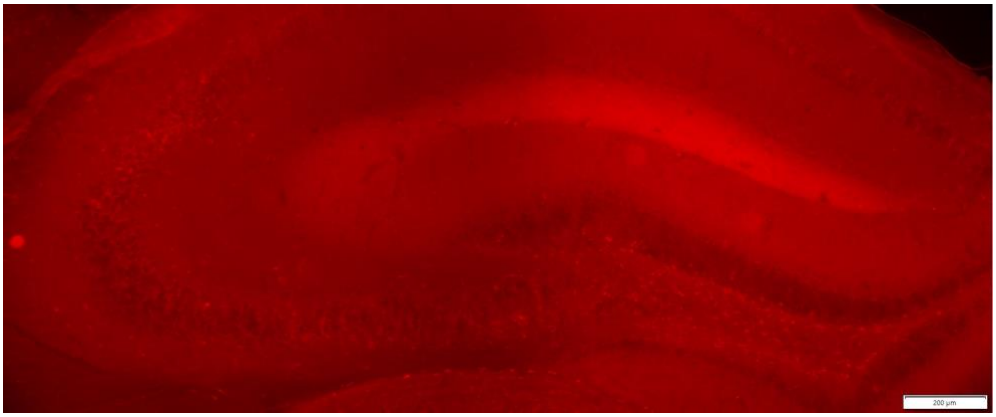


Figure 8B: Control Left Hippocampus Immunofluorescence



CHAPTER FOUR

Discussion

Key Findings

Alcohol-use disorders (AUDs) are a major public health concern, with devastating consequences for individuals and society as a whole. Binge drinking, in particular, has been identified as a significant risk factor for the development of AUDs, as well as a host of negative health outcomes, including fear learning impairment. Despite the high burden of prevalent binge drinking and alcohol-related fatalities, more is needed to be known about the neural mechanisms underpinning the relatedness of high, chronic alcohol consumption to fear learning cognitive function. The present study aimed to further investigate behavioural neural mechanisms underlying binge ethanol consumption and its effect on fear learning in mice with special emphasis on the hippocampus. To this end, we employed a combination of behavioural and immunohistochemical techniques to examine the differences in neural output that occurred following ethanol exposure. Previous studies – either using alcohol vapor chambers, operant conditioning to alcohol, or ethanol injections – have exhibited hippocampal-specific impairments in fear learning in rodent models exposed to chronic alcohol (Holmes et al., 2012, Weitemier and Ryabinin, 2003, Stephens et al., 2001). However, while other studies examining the effect of chronic alcohol on fear learning have similarly shown an interaction between elevated ethanol intake and fear learning impairments, few have utilized a free bottle choice model such as the one used in this study whereby the mice were at liberty to choose between alcohol and water on given days.

Our behavioural results indicate that mice exposed to the intermittent-access ethanol protocol exhibited a significant increase in their ethanol consumption compared to control mice which can be qualified as binge-like behaviour. More interestingly, these ethanol-exposed, binge-like mice displayed impaired, hippocampal-based fear learning, as supported by decreased freezing behaviour across two cohorts during the trace fear conditioning task and subsequent extinction tests. Past research studying the effect of chronic alcohol on the hippocampus have similarly elucidated impairments in trace fear conditioning and spatial memory in murine models (Weitemier, 2003, McDaniel, 1990). Because an impaired fear learning effect was seen in our trace conditioning protocol, which necessitates the use and activation of the hippocampus and LTP-related neural circuit, this finding supports our hypothesis of chronic, binge-like drinking having negative effects on normative fear learning. To further qualify this claim, immunohistochemistry was needed to better visualize hippocampal differences within the effect seen.

In examining the underlying neural mechanisms of these behavioural effects, we performed immunohistochemical analysis of perfused brain tissue from ethanol-exposed IA and control H_2O mice. With focus on the hippocampus, a key brain region involved in fear learning and long-term fear forming memories, our results indicated higher hippocampal activation in IA-exposed mice than in water controls. The significantly larger c-Fos puncta count seen in the binge-like mice supported our overarching hypothesis; however, it was interesting to increased hippocampal activation in IA mice considering the impaired fear learning, and because no conclusion can be drawn to the state of their hippocampal beta-dynorphin activation, nothing can be concluded about dynorphin relatedness to the behavioural outputs seen.

Limitations of the Study

The present study provides valuable insights into the potential impact of chronic, elevated ethanol on fear learning. Nevertheless, certain limitations must be acknowledged. Firstly, while the animal model used in this study provides a useful tool for investigating the neural underpinnings of fear learning, it may not fully capture the complexities of AUD in human populations. Additionally, the duration and extent of AUD exposure in human populations are likely to vary widely and across different socio-cultural groups, making it difficult to generalize findings from animal studies to human populations. Therefore, caution is advised in extrapolating findings from this study on a binge-like models to humans with AUD. Additionally, the sample size in this study consisted of a total subject population of 48 mice, which is within and above-average of literature standards of this sort of research. Nevertheless, larger power and replicability is always needed to provide more definitive evidence regarding the findings such as those entailed in this study. Secondly, our study focused primarily on the effects of chronic ethanol acute withdrawal and its effect on the hippocampus in light of fear learning. We were able to visualize an effect of higher c-Fos puncta, or neural activation, in critical fear-forming regions of the hippocampi of IA mice. However, chronically high ethanol consumption and its subsequent effect on fear learning in terms of beta-dynorphin differences cannot be derived from the results of this work. It yet remains unclear how binge-like ethanol exposure may impact beta-dynorphin output in the hippocampus, and further research in warranted in this regard. Thirdly, our study examined the effect of intermittent access ethanol on fear learning in male mice, without considering the potential impact of this protocol on female

mice. AUD preclinical research is predominately mired in its ability to better understand, and accrue reliable and valid transability, ethanol's effect on female sex. It should be noted that further research of the potential sex differences of this study is imperative to ameliorate our holistic understanding of how fear learning can be impacted by chronic ethanol. In summary, while this study provides important insights into the impact of binge-like drinking on fear learning, these aforementioned limitations must be considered when interpreting the results. Further research is needed to fully elucidate the impact of AUD on fear learning and other behavioural and neurochemical processes.

Future Directions

Due to the limitations of this study and the need for valid and replicable follow-up research, future directions must be undertaken. Firstly, because visualization of beta-dynorphin could not be elucidated to corroborate the hypothesis that elevated levels of this neuropeptide are correlated to ethanol-induced fear learning differences, more needs to be done to qualify such claim. A chemogenetic approach utilizing DREADDs (Designer Receptor Exclusively Activated for Designer Drugs) would be an optimal way to better investigate the relationship between beta-dynorphin and fear learning differences in IA mice. Pdyn cre-line mice that were injected with an adeno-associated virus containing cre-recombinase to inhibit Pdyn (AAV5-hSyn-DIO-hM4Di-mCherry) would be necessary to visualize a potential regression of those mice back to normative freezing behaviour, and thus qualifying the beta-dynorphin hypothesis should it prove evidence.

Another future direction for this study would be to replicate the trace conditioning fear protocols with the use of a kappa-opioid receptor antagonist in the experimental IA

group, which should theoretically block the hyper-activation of hippocampal CA1, CA3, and dentate gyrus. This should thus help retain LTP in binge-like drinking mice and one would expect no significant differences between groups. Previous preclinical research has shown KOR antagonists, like naloxone, to block hippocampally-based learning impairments in elevated beta-dynorphin models (McDaniel 1990). It would be fruitful to see positive correlations between the administration of a KOR antagonist drug, such as naloxone which is already a commonly prescribed medication for AUD, and a regression to baseline/control levels of fear learning within the same experimental context as our study. This would be an important corroborator for the expected results of the current study.

Conclusion

Our findings shed light on the detrimental impact of alcohol-use disorder on fear learning in a male mice model as well as activational neural differences in the underlying mechanisms involved. Our study demonstrated that ethanol exposure, particularly in the context of binge-like drinking, impairs the natural fear response in mice, which may have implications for human behaviour in fear-related situations. It is our hope that these results contribute to the collective effort in alcohol addiction research and provide benefit for future translational studies investigating the impact of alcohol on learning and memory.

REFERENCES

National Highway Traffic Safety Administration (NHTSA). Traffic Safety Facts 2020 Data: Alcohol-Impaired Driving (Report No DOT HS 813 294). Washington, DC: U.S. Department of Transportation, National Highway Traffic Safety Administration, National Centre for Statistics and Analysis; April 2022.

Alcohol Use Disorder (AUD) in the United States | National Institute on Alcohol Abuse and Alcoholism (NIAAA). (n.d.). Retrieved April 2, 2023, from https://www.niaaa.nih.gov/alcohols-effects-health/alcohol-topics/alcohol-facts-and-statistics/alcohol-use-disorder-aud-united-states

Ballenger, J. C., & Post, R. M. (1978). Kindling as a model for alcohol withdrawal syndromes. *The British Journal of Psychiatry: The Journal of Mental Science*, *133*, 1–14. https://doi.org/10.1192/bjp.133.1.1

Becker, H. C. (1998). Kindling in Alcohol Withdrawal. *Alcohol Health and Research World*, *22*(1), 25–33.

Bilkei-Gorzo, A., Erk, S., Schurmann, B., Mauer, D., Michel, K., Boecker, H., Scheef, L., Walter, H., & Zimmer, A. (2012). Dynorphins Regulate Fear Memory: From Mice to Men. *Journal of Neuroscience*, *32*(27), 9335–9343. https://doi.org/10.1523/JNEUROSCI.1034-12.2012

Carey, A. N., Lyons, A. M., Shay, C. F., Dunton, O., & McLaughlin, J. P. (2009). Endogenous κ Opioid Activation Mediates Stress-Induced Deficits in Learning and Memory. *The Journal of Neuroscience*, *29*(13), 4293–4300. https://doi.org/10.1523/JNEUROSCI.6146-08.2009

CDC. (2022, July 11). *Excessive Alcohol Use*. Centers for Disease Control and Prevention. https://www.cdc.gov/chronicdisease/resources/publications/factsheets/alcohol.htm

Dallman, M. F., Akana, S. F., Bradbury, M. J., Strack, A. M., Hanson, E. S., & Scribner, K. A. (1994). Regulation of the hypothalamo-pituitary-adrenal axis during stress: Feedback, facilitation and feeding. *Seminars in Neuroscience*, *6*(4), 205–213. https://doi.org/10.1006/smns.1994.1027

Davis, M., & Whalen, P. J. (2001). The amygdala: Vigilance and emotion. *Molecular Psychiatry*, *6*(1), 13–34. https://doi.org/10.1038/sj.mp.4000812

Esel, E., Sofuoglu, S., Aslan, S. S., Kula, M., Yabanoglu, I., & Turan, M. T. (2001). PLASMA LEVELS OF BETA-ENDORPHIN, ADRENOCORTICOTROPIC HORMONE AND CORTISOL DURING EARLY AND LATE ALCOHOL WITHDRAWAL. *Alcohol and Alcoholism*, *36*(6), 572–576. https://doi.org/10.1093/alcalc/36.6.572

Gewirtz, J. C., McNish, K. A., & Davis, M. (2000). Is the hippocampus necessary for contextual fear conditioning? *Behavioural Brain Research*, *110*(1–2), 83–95. https://doi.org/10.1016/s0166-4328(99)00187-4

Gilpin, N. W., Richardson, H. N., & Koob, G. F. (2008). Effects of CRF1-receptor and opioid-receptor antagonists on dependence-induced increases in alcohol drinking by alcohol-preferring (P) rats. *Alcoholism, Clinical and Experimental Research*, *32*(9), 1535–1542. https://doi.org/10.1111/j.1530-0277.2008.00745.x

Grover, D., Chen, J.-Y., Xie, J., Li, J., Changeux, J.-P., & Greenspan, R. J. (2022). Differential mechanisms underlie trace and delay conditioning in Drosophila. *Nature*, *603*(7900), Article 7900. https://doi.org/10.1038/s41586-022-04433-6

Holley, A. J., & Lugo, J. N. (2016). Effects of an acute seizure on associative learning and memory. *Epilepsy & Behavior: E&B*, *54*, 51–57. https://doi.org/10.1016/j.yebeh.2015.11.001

Holmes, A., Fitzgerald, P. J., MacPherson, K. P., DeBrouse, L., Colacicco, G., Flynn, S. M., Masneuf, S., Pleil, K. E., Li, C., Marcinkiewcz, C. A., Kash, T. L., Gunduz-Cinar, O., & Camp, M. (2012). Chronic alcohol remodels prefrontal neurons and disrupts NMDAR-mediated fear extinction encoding. *Nature Neuroscience*, *15*(10), 1359–1361. https://doi.org/10.1038/nn.3204

Hwa, L. S., Chu, A., Levinson, S. A., Kayyali, T. M., DeBold, J. F., & Miczek, K. A. (2011). Persistent Escalation of Alcohol Drinking in C57BL/6J Mice With Intermittent Access to 20% Ethanol. *Alcohol: Clinical and Experimental Research*, *35*(11), 1938–1947. https://doi.org/10.1111/j.1530-0277.2011.01545.x

Hwa, L. S., Holly, E. N., DeBold, J. F., & Miczek, K. A. (2016). Social stress-escalated intermittent alcohol drinking: Modulation by CRF-R1 in the ventral tegmental area and accumbal dopamine in mice. *Psychopharmacology*, *233*(4), 681–690. https://doi.org/10.1007/s00213-015-4144-2

Hwa, L. S., Neira, S., Flanigan, M. E., Stanhope, C. M., Pina, M. M., Pati, D., Hon, O. J., Yu, W., Kokush, E., Calloway, R., Boyt, K., & Kash, T. L. (2020). Alcohol drinking alters stress response to predator odor via BNST kappa opioid receptor signaling in male mice. *ELife*, *9*, e59709. https://doi.org/10.7554/eLife.59709

Hwa, L. S., Shimamoto, A., Kayyali, T., Norman, K. J., Valentino, R. J., DeBold, J. F., & Miczek, K. A. (2016). Dissociation of μ-opioid receptor and crf-r1 antagonist effects on escalated ethanol consumption and mpfc serotonin in c57bl/6j mice: Drn and intermittent ethanol. *Addiction Biology*, *21*(1), 111–124. https://doi.org/10.1111/adb.12189

Kim, W. B., & Cho, J.-H. (2020). Encoding of contextual fear memory in hippocampal–amygdala circuit. *Nature Communications*, *11*(1), Article 1. https://doi.org/10.1038/s41467-020-15121-2

Knoll, A. T., & Carlezon, W. A. (2010). Dynorphin, stress, and depression. *Brain Research*, *1314C*, 56. https://doi.org/10.1016/j.brainres.2009.09.074

Lê, A. D., Ko, J., Chow, S., & Quan, B. (1994). Alcohol consumption by C57BL/6, BALB/c, and DBA/2 mice in a limited access paradigm. *Pharmacology, Biochemistry, and Behavior*, *47*(2), 375–378. https://doi.org/10.1016/0091-3057(94)90026-4

Limoges, A., Yarur, H. E., & Tejeda, H. A. (2022). Dynorphin/kappa opioid receptor system regulation on amygdaloid circuitry: Implications for neuropsychiatric disorders. *Frontiers in Systems Neuroscience*, *16*, 963691. https://doi.org/10.3389/fnsys.2022.963691

Lugo, J. N., Smith, G. D., & Holley, A. J. (2014). Trace Fear Conditioning in Mice. *Journal of Visualized Experiments : JoVE*, *85*, 51180. https://doi.org/10.3791/51180

McDaniel, K. L., Mundy, W. R., & Tilson, H. A. (1990). Microinjection of dynorphin into the hippocampus impairs spatial learning in rats. *Pharmacology, Biochemistry, and Behavior*, *35*(2), 429–435. https://doi.org/10.1016/0091-3057(90)90180-p

Raber, J., Arzy, S., Bertolus, J. B., Depue, B., Haas, H. E., Hofmann, S. G., Kangas, M., Kensinger, E., Lowry, C. A., Marusak, H. A., Minnier, J., Mouly, A.-M., Mühlberger, A., Norrholm, S. D., Peltonen, K., Pinna, G., Rabinak, C., Shiban, Y., Soreq, H., … Boutros, S. W. (2019). Current understanding of fear learning and memory in humans and animal models and the value of a linguistic approach for analyzing fear learning and memory in humans. *Neuroscience & Biobehavioral Reviews*, *105*, 136–177. https://doi.org/10.1016/j.neubiorev.2019.03.015

Sandin, J., Nylander, I., Georgieva, J., Schött, P. A., Ögren, S. O., & Terenius, L. (1998). Hippocampal dynorphin B injections impair spatial learning in rats: A κ-opioid receptor-mediated effect1Parts of these results were presented at the International Narcotics Research Conference 1995, St Andrews, U.K.1. *Neuroscience*, *85*(2), 375–382. https://doi.org/10.1016/S0306-4522(97)00605-2

Silva, B. A., Burns, A. M., & Gräff, J. (2019). A cFos activation map of remote fear memory attenuation. *Psychopharmacology*, *236*(1), 369–381. https://doi.org/10.1007/s00213-018-5000-y

Stephens, D. N., Brown, G., Duka, T., & Ripley, T. L. (2001). Impaired fear conditioning but enhanced seizure sensitivity in rats given repeated experience of withdrawal

from alcohol. *European Journal of Neuroscience*, *14*(12), 2023–2031. https://doi.org/10.1046/j.0953-816x.2001.01824.x

Wagner, J. J., Terman, G. W., & Chavkin, C. (1993). Endogenous dynorphins inhibit excitatory neurotransmission and block LTP induction in the hippocampus. *Nature*, *363*(6428), 451–454. https://doi.org/10.1038/363451a0

Weitemier, A. Z., & Ryabinin, A. E. (2003). Alcohol-induced memory impairment in trace fear conditioning: A hippocampus-specific effect. *Hippocampus*, *13*(3), 305–315. https://doi.org/10.1002/hipo.10063

Yu, W., Hwa, L. S., Makhijani, V. H., Besheer, J., & Kash, T. L. (2019). Chronic inflammatory pain drives alcohol drinking in a sex-dependent manner for C57BL/6J mice. *Alcohol*, *77*, 135–145. https://doi.org/10.1016/j.alcohol.2018.10.002