Effects of the Dynorphin/Kappa Opioid Receptor System on Stress Reactions After Intermittent Alcohol Drinking and Withdrawal in Female C57BL/6J Mice

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ABSTRACT

Alcohol use and abuse disorders are closely tied with stress, depression, and anxiety by disrupting the body's normal hormonal stress responses. Specifically, the kappa opioid receptor (KOR)/dynorphin (DYN) system in the central nervous system is an important regulator of anxiety, depression, and addiction, and has been shown to contribute to the negative reinforcing and pro-depressive effects of alcohol. Previous studies have shown that norbinaltophimine (norBNI), a long-acting KOR antagonist, reduces depressive phenotypes and maladaptive stress reactions in alcohol-dependent male C57BL/6J mice experiencing protracted withdrawal. This relationship has not been studied in female mice. We investigated the role of the DYN/KOR system in alcohol-dependent female C57BL/6J mice experiencing protracted alcohol withdrawal. To facilitate voluntary drinking and alcohol dependence, we exposed female C57BL/6J mice to intermittent access to alcohol (IA), a two-bottle paradigm, which was rotated every 24 hours between only water, and simultaneous access to an ethanol solution (20% w/v) and water. Over a six-week drinking period, ethanol preference increased and the female mice developed a preference for ethanol over water. Stress responses were tested after a period of protracted alcohol withdrawal using the repeated forced swim test (FST) and the exposure to predator odor test. In FST, we discovered that treatment with norBNI in both alcohol and water drinkers increased immobility duration, an adaptive response to conserve energy. However, there were no significant differences in any behaviors between alcohol and water drinkers in the FST, implying that alcohol drinking followed by protracted withdrawal may not impact forced swim stress reactions in female mice. In the predator odor test, alcohol-drinking females showed more anxiety-like baseline behavior than water-drinking females. Additionally, norBNI increased defensive burying regardless of alcohol history. Consequently, these findings suggest norBNI

could play a role in learned adaptations by preventing the depressive effects of the DYN/KOR system. Taken together with previous data from male mice, the implications of these results could ultimately help develop effective behavioral or pharmacological treatments for alcohol addiction and stress.

INTRODUCTION

Alcohol use disorders, including alcohol use and dependence, are prevalent health concerns for the adult population as they are the third leading cause of preventable death (Walker et. al., 2012; Grant et. al., 2004; Mokdad et. al., 2004). Alcohol use disorders are closely tied with other psychiatric illnesses such as stress, anxiety, and depression, and can create a cycle of increased alcohol usage and relapse behavior as "self-medication," which can further escalate negative withdrawal effects leading to addiction (Gilligan, Reich, & Cloninger, 1987; Khantzian, 1990; Markou, Kosten, & Koob, 1998). Although alcohol may serve as a temporary stress reliever and anxiety reducing agent, it has been shown that chronic alcohol exposure and withdrawal can themselves act as stressors, disrupting the hypothalamic–pituitary–adrenocortical (HPA) axis, the body's hormonal stress response (Becker, 2012). This disruption can cause maladaptive stress coping behaviors, which can contribute to relapse and other dangerous outcomes for the individual (Hwa et. al., 2019). Animals, such as mice and rats, are often used to model chronic drinking and withdrawal because they will voluntarily consume unsweetened alcohol, even in the presence of water. Likewise, animal models allow us to achieve a greater understanding of the neurobiology involved in alcohol use and subsequent stress, due to the similarities between human and rodent neuronal mechanisms. Ultimately, these models are important to study in order to develop effective treatments for anxiety and alcohol addiction.

Kappa opioid receptors (KORs) in the central nervous system are important regulators of anxiety, depression, and addiction (Crowley & Kash, 2015). Dynorphin (DYN) is the endogenous ligand for KORs and the DYN/KOR system has been shown to contribute to the negative reinforcing effects of alcohol (Walker & Koob, 2008). Additionally, acute and chronic alcohol exposure result in an upregulation of the DYN/KOR system. One cause of addiction and

relapse could be the DYN/KOR system's pro-depressive effects, resulting in a perceived need for "self-medication" with alcohol. In fact, many studies with animal models have shown that the DYN/KOR system is largely responsible for increased alcohol drinking when stressed. It has similarly been demonstrated to contribute to depressive phenotypes and maladaptive stress reactions in alcohol-dependent male mice (Crowley & Kash, 2015; Hwa et. al., 2019). These relationships were elucidated using KOR antagonists, such as norbinaltorphimine (norBNI). However, KOR function disparately impacts post-drinking affective state and addictive behavior in male and female mice (Chartoff & Mavrikaki, 2015). Thus, it is unknown whether the DYN/KOR system would be responsible for similar maladaptive stress behaviors in alcohol-dependent female mice as in male mice. We investigated this relationship by determining if alcohol-induced, maladaptive stress coping mechanisms in female mice would be reverted after treatment with a long-acting KOR antagonist, norBNI.

Female C57BL/6J mice were used in this study because the C57BL/6J strain voluntarily consumes the most alcohol per unit body weight and shows the highest ethanol preference. Furthermore, female C57BL/6J mice have a higher ethanol preference compared to males, and on average consume more alcohol per unity body weight (Hwa et. al., 2011). Mice were given intermittent access to ethanol, a protocol proven to induce high volumes of voluntary drinking due to the effects of acute withdrawal (Hwa et. al., 2011). Stress reactions were observed and quantified after protracted ethanol withdrawal through the exposure to predator odor test and the repeated forced swim test (FST). We predicted that norBNI would cause alcohol-dependent female mice to react to anxiety-inducing stimuli more like water-drinking female mice, thereby, rescuing any alcohol-induced maladaptive effects.

MATERIALS AND METHODS

Animals and Housing:

Female C57BL/6J mice (n = 24) (Jackson Laboratories, Bar Harbour, ME) were initially housed at 8 weeks old in groups of four or five mice per cage. One week prior to intermittent alcohol (IA) access, the mice were habituated to a 12-hour reversed light/dark cycle with lights off at 07:00. The mice were single-housed in polycarbonate cages with corn cob pellet bedding, food (Prolab Isopro RMH 3000, LabDiet, St. Louis, MO), water, and a nestlet during this time. Mice had ad-libitum access to food, tap water, and a clean cage throughout the experiment with the exclusion of during the stress tests (Hwa et. al., 2011). All procedures were conducted in accordance with the NIH Guide to Care and Use of Laboratory Animals guidelines (Institute for Animal Laboratory Research, 2011).

Ethanol Intake Procedures:

Ethanol solutions (20% w/v) were prepared by diluting 95% ethyl alcohol. Three days before introduction of the alcohol, the alcohol drinking cohort (n = 12) was exposed to two centrifuge tubes, containing only tap water, to acclimate to drinking from the two-bottle choice arrangement. Fluids were presented on the cage lid in 100-mL plastic centrifuge tubes with rubber stoppers and stainless steel sippers (Hwa et. al. 2011).

The mice were allowed intermittent access to alcohol for a period of six weeks, or eighteen drinking sessions, to facilitate the transition to alcohol dependence. Mice were exposed to one ethanol bottle and one water bottle, for periods of 24 hours at a time. After 24 hours, the ethanol bottle was removed, and 2 bottles of water were then available for the mouse instead. Both bottles were weighed to the nearest hundredth of a gram before presentation of ethanol and

water, and weighed again 24 hours after the initial presentation. Every other day, ethanol was reintroduced three hours into the dark cycle, and placed on a different side with respect to the water bottle than in the previous drinking session. This was done to eliminate a potential habituated side preference. The 20% w/v ethanol solution, without sweeter, was available every Monday, Wednesday, and Friday, and only water was offered the remaining days of the week [Supplemental Figure 1]. A 24-hour rotation of two-bottle choice access to ethanol and water simulated voluntary drinking conditions in humans.

To control for evaporation or fluid spillage from bottles, we calculated the loss of fluid in a cage without an animal, for each administration of ethanol. This loss was subtracted from the difference in liquid volume over the same 24-hour drinking period to determine the ethanol intake over 24 hours per mouse. Ethanol preference was calculated as ethanol intake (mL) divided by total intake of ethanol and water (mL). Mice were weighed weekly to the nearest tenth of a gram to ensure increase of body mass due to alcohol intake (Hwa et. al. 2011).

Behavioral Assays after Ethanol Drinking:

Experiment 1 – Forced-Swim Test (FST):

After six weeks of access to intermittent alcohol, the mice experienced three days of protracted withdrawal with only access to water. Then, individual mice were placed into a transparent, acrylic cylinder (30 cm height x 20 cm diameter) that was filled with 23-25°C water. Swimming and immobility were observed and recorded using an overhead camera for ten minutes. At the end of the test, mice carefully were removed from the water, dried with a dry towel, and returned to their home cages (Can et. al 2012).

Sixteen hours prior to second exposure to the forced swim test, half of the mice (IA n=6, H_2O n=6) were injected intraperitoneally (IP) with 10mL/kg of 5mg/kg norbinaltorphimine (norBNI) (Cat no. 0347, Tocris), a long-acting kappa opioid receptor (KOR) antagonist. The other half (IA n=6, H_2O n=6) was injected IP with the same quantity of an isotonic saline solution. Subsequently, all mice underwent a second exposure to the forced-swim test, which occurred 72 hours after the first forced-swim trial and 16 hours after injections. Video recordings of both swim trials for each mouse were started before the mice were placed in the water and stopped after 10 minutes of recording. Using the video recording, latency to immobility in seconds was hand-scored by a blind observer. Duration of mobility (swimming) and duration of immobility were electronically scored by Ethovision XT13 (Noldus, The Netherlands).

Experiment 2 – Predator Odor Exposure:

Exposure to predator odor occurred after FST, so mice were in protracted withdrawal (7-10 days) and had already received injections of saline or norBNI prior to this test. The predator odor was a synthetic scent derived from fox feces, trimethylthiazoline (TMT), and has been shown to induce stress behaviors in male C57BL/6J mice. Before the introduction of TMT, mice were habituated for 10 minutes to an unscented cotton swab held vertically in a plastic stand that was placed in the corner of their rectangular home cage. After this baseline trial, 2.5 µL of TMT was applied to the cotton tip and left in the cage with the mouse for 10 minutes [Supplemental Figure 2]. The baseline and TMT trials were recorded on video and quantified by Ethovision XT13 for time spent in contact with the object and in the far corners of the cage. Duration of defensive burying was hand-scored by a blind observer (Hwa et. al., 2019).

Experiment 3 - Peanut Oil Exposure

In order to analyze whether intermittent alcohol exposure had an effect on olfaction, male C57BL/6J mice (n=31) were exposed to peanut oil, an appetitive scent, in the home cage. Intermittent alcohol mice (n=16) were in protracted withdrawal (7-10 days without ethanol) during testing. Similarly, to the predator odor exposure test, a baseline trial was run for 10 minutes with an unscented cotton swab object, before the introduction of peanut oil. After the baseline trial, 2.5 µL of peanut oil (Harris Teeter, Matthews, NC) was applied to the cotton tip and left in the cage with the mouse for 10 minutes. The baseline and peanut oil trials were recorded on video and quantified by Ethovision XT13 for time spent in contact with the object and in the far corners of the cage.

Statistical Analyses

The repeated forced swim test data were analyzed through three-way repeated measures ANOVA to assess the impact of norBNI vs. saline, IA exposure vs. H_2O control, and swim 1 vs. swim 2, on the dependent variables of latency to immobility, duration of swimming, and duration of immobility. For the predator odor test, a two-way repeated measures ANOVA was used to analyze the behavioral data and determine the influence of the drug, norBNI, and influence of alcohol on time spent in contact with object, time spent in far corners, and duration of defensive burying. If significant main effects were found in the ANOVA analysis, the Holm-Sidak post hoc tests were subsequently used to analyze the differences between independent variables. An unpaired t-test was used to assess differences in coping behavior between object habituation and exposure to TMT. The presented values are mean ± standard error of the mean (SEM) with an $\alpha = 0.05$.

RESULTS

Ethanol Preference and Intake Increased as a Result of Intermittent Access to Alcohol:

Figure 1A: Intermittent Ethanol Intake

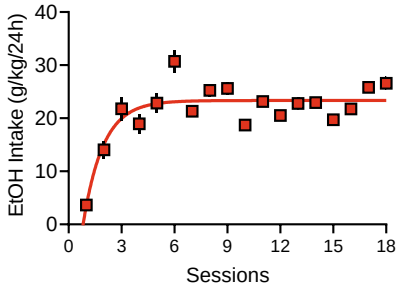
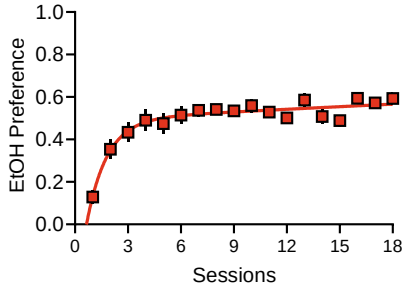


Figure 1B: Ethanol Preference/ 24 hr



Female C57BL/6J mice (n = 12) were given intermittent access to 20% ethanol for 3 drinking sessions a week, for 6 weeks (total 18 sessions). Figure 1A shows that after one week of IA exposure, ethanol intake increased to an average of 22 g/kg per 24 hour drinking session. Likewise, ethanol preference drastically increased from an average of 0.1 to over 0.4 after the first week, and steadily continued increasing in the following weeks to an average of 0.6 [Figure 1B]. This indicates that over the period of 6 weeks of intermittent access to alcohol, the female mice developed a preference to alcohol over water on average [Figure 1B].

Forced Swim Test:

The cohort of female C57BL/6J mice (n = 12 IA, n = 12 H_2O) were exposed to the forced swim test twice (S1 and S2) and scored for latency to immobility [Figure 2A], duration of immobility [Figure 2B], and the duration of swimming (or high mobility) [Figure 2C]. We considered immobility during the forced swim test to be a learned adaptive behavior in order to conserve energy in the absence of an escape. We hypothesized norBNI would rescue any alcohol-induced maladaptive responses in female mice and decrease their latency to immobility and swimming duration, and increase their duration of immobility.

Figure 2A: Latency to Immobility

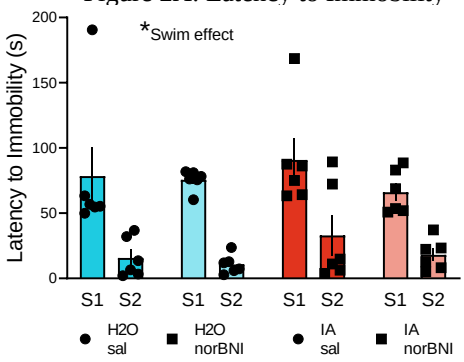


Figure 2A:
Three-way ANOVA
Main effect of swim F(1,20)=89.80, *p<0.001

Figure 2B: Duration of Immobility

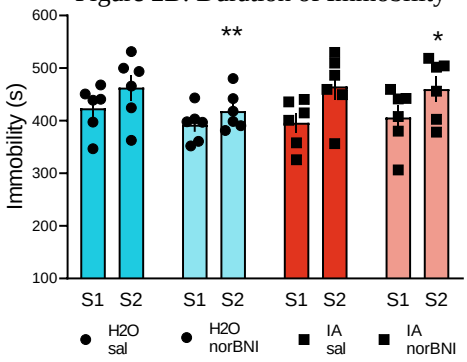


Figure 2B:
Three-way ANOVA
Main effect of swim F(1,20)=24.56, *p<0.001
Post-hoc multiple comparisons
H2O norBNI S1 vs S2: t(20)=3.64, **p=0.0065
IA norBNI S1 vs S2: t(20)=2.84, *p=0.0397

Figure 2C: Duration of Swimming

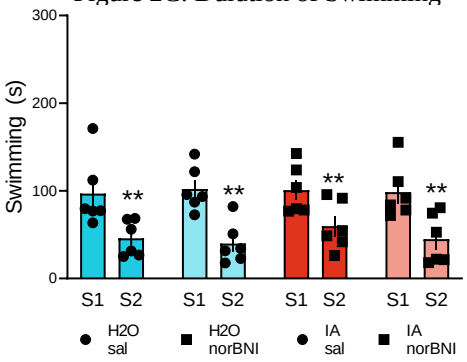


Figure 2C:
Three-way ANOVA
Main effect of swim F(1,20)=85.14, *p<0.001
Post-hoc multiple comparisons
H2O saline S1 vs S2: t(20)=4.52, **p=0.0008
H2O norBNI S1 vs S2: t(20)=5.53, **p<0.0001
IA saline S1 vs S2: t(20)=3.65, **p=0.0064
IA norBNI S1 vs S2: t(20)=4.76, **p=0.0005

Latency to Immobility Decreased and Immobility Duration Increased for all Mice from Swim 1 to Swim 2:

A three-way repeated measures ANOVA was performed for the dependent variable of latency to immobility and independent variables of swim trial, IA exposure, and norBNI injection. It was found that previous exposure to FST caused a decrease in latency to immobility [Figure 2A] for all mice from swim 1 to swim 2 ([F(1,20)=89.80, p<0.001]). A three-way repeated measures ANOVA was performed for duration of immobility [Figure 2B] with the aforementioned independent variables. There was a significant main effect of swim trial ([F(1,20)=24.56, p<0.001]). Thus, previous exposure to the stressor caused all mice to become immobile faster and for a longer period of time during the second exposure as compared to the first.

NorBNI Increased Immobility Duration for All Mice Regardless of Alcohol History:

Post-hoc analysis revealed that treatment with norBNI significantly increased immobility in swim 2 in both water drinkers ([t(20) = 3.64, p<0.05)] and ethanol drinkers ([t(20)=2.84, p<0.05]). Similarly, a three-way repeated measures ANOVA analyzing the duration of

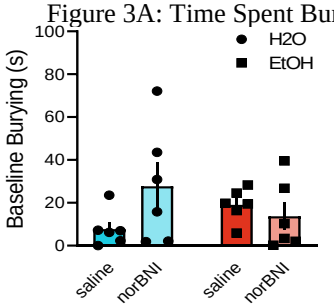
swimming [Figure 2C] as the dependent variable showed a significant main effect of the swim trial ([F(1,20)=85.14, p<0.001]). All groups showed a significant decrease in duration of swimming from the first FST trial to the second, regardless of alcohol or norBNI exposure.

Alcohol Drinking had No Effect on Forced Swim Test in Females:

There were no significant differences found between alcohol drinking and water drinking animals treated with saline (n=6 IA, n=6 H_2O) in any of the measured stress behaviors for the forced swim test [Figure 2A, Figure 2B, Figure 2C].

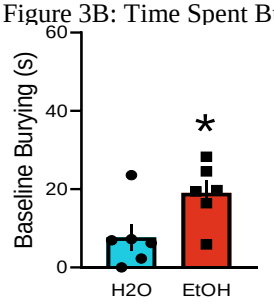
Exposure to Predator Odor:

Figure 3A: Time Spent Burying



Two-way ANOVA
F(1,20)=3.44, p=0.07

Figure 3B: Time Spent Burying



Unpaired t-test
t(10)=2.46, *p=0.0338

Alcohol-drinking Mice Showed Higher Defensive Burying than Water-drinkers during Baseline:

Immediately preceding the addition of TMT, mice (n = 24) were tested in their home cages with a foreign object, to which TMT would later be applied. Stress-coping behaviors including duration of defensive burying, time spent in far corners of the cage, and time spent in contact with object were all observed and quantified. We predicted that there would be no differences in behavior between any of the subgroups (n=6/group) during the baseline trial due to the absence of an applied stressor. However, there was a trend towards an interaction between alcohol history and norBNI treatment on defensive burying during the baseline period ([F(1,20)=3.44, p=0.07; Figure 3A]). An unpaired t-test showed that, at baseline without TMT

exposure, alcohol drinking female mice exhibited increased defensive burying than water drinking females ([t(10)=2.46, p<0.05; Figure 3B]).

Figure 4A: Time Spent in Contact with TMT

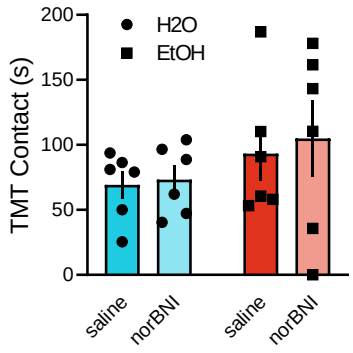


Figure 4B: Time Spent in Far Corners

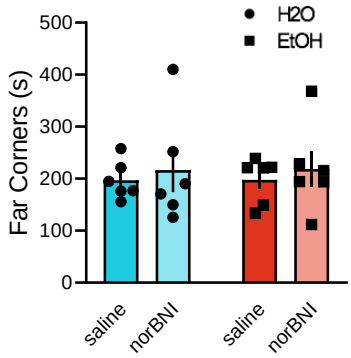


Figure 4C: Duration of Defensive Burying

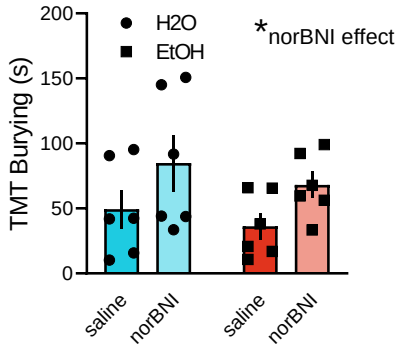


Figure 4C:
Two-way ANOVA
Main effect of drug F(1,20)=5.11, *p=0.0351

NorBNI Increased Burying Behavior Regardless of Alcohol History in the Presence of TMT:

The female C57BL/6J mice (n=24) were exposed to TMT in their home cages after IA mice (n=12) had experienced 7-10 days of protracted ethanol withdrawal. The same measures for stress-coping behaviors were used in the TMT trial as with the baseline trial. We hypothesized that treatment with norBNI would cause alcohol-drinking animals to cope with the predator odor more appropriately by spending more time avoiding TMT and increasing defensive burying, than without treatment. Time spent in contact with TMT and time spent in far corners did not produce any significant effects as analyzed with two-way ANOVAs [Figure 4A, Figure 4B]. A repeated measures two-way ANOVA showed a significant increase in defensive burying behavior [Figure 4C] due to norBNI ([F(1,20) = 5.11, p <0.05]), but no significant different between IA and H_2O mice.

Exposure to Peanut Oil:

Figure 5A: Time Spent in Contact with Peanut Oil

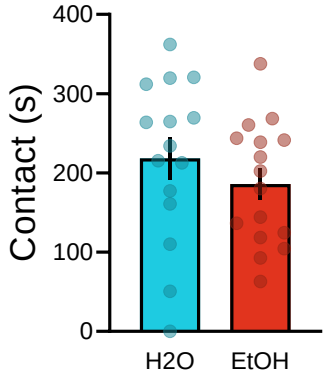
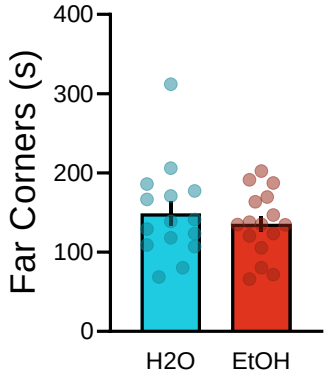


Figure 5B: Time Spent in Far Corners



Alcohol Male Mice Contacted Peanut Oil as Often as Peanut Controls

We aimed to determine whether or not mice olfaction was impaired by alcohol exposure, thereby potentially affecting the exposure to predator odor test results. To accomplish this, we monitored the behaviors of male C57BL/6J mice (n=16 IA, n=15 H_2O) in the presence of peanut oil, an appetitive scent to mice. We hypothesized that there were no olfactory deficits in alcohol exposure mice and, thus, there would be no behavioral differences between the alcohol drinkers and controls in the presence of peanut oil. Our hypothesis was supported by the results as there were no significant differences between the variable and control groups in time spent in contact with the peanut oil or time spent avoiding peanut oil [$t(29)=0.99$, $p=0.33$, Figure 5A, $t(29)=0.73$, $p=0.47$, Figure 5B].

DISCUSSION

The results of this study confirmed that female C57BL/6J mice, given intermittent access to alcohol for six weeks, will voluntarily consume increasingly larger quantities of alcohol [Figure 1A], increasing their preference to an average of 60% alcohol over water during the 24 hour drinking sessions [Figure 1B]. These findings are consistent with previous studies (Hwa et. al., 2011). The intermittent access to ethanol protocol simulated the human condition since water was consistently available to the mice, and the periods of acute withdrawal may have driven the mice to increase their alcohol intake to the point of heavy drinking during the drinking sessions. After six weeks, access to alcohol was removed and mice experienced 3 days of abstinence before the first trial of the repeated forced swim test. The second trial occurred 3 days later and 16 hours after mice were injected with norBNI.

The data showed that latency to immobility significantly decreased for all experimental groups, regardless of alcohol or norBNI treatment, between swim 1 and swim 2, with no significant differences between the groups [Figure 2A]. This could be explained by learned adaptive response from the first swim to the second. In this study, immobility during FST was considered to be an adaptive response to conserve energy in the absence of an escape. Other studies such as Porsolt et. al., 1977 suggest that immobility during the FST is a sign of depressive-like behavior and behavioral despair, indicating that the mouse is no longer trying to escape the stressful condition. However, further research has shown that immobility when faced with this acute stressor is actually a behavioral adaptation for survival (Molendijk et. al., 2015; Commons et al., 2017), which is consistent with the results of this study. Furthermore, the data showed that norBNI significantly increased immobility duration in both water and alcohol drinkers in the second trial of FST [Figure 2B], suggesting norBNI could play a role in learned

adaptations by preventing the depressive effects of the DYN/KOR system. There were no significant differences between alcohol and water controls in any of the measured FST behaviors, which could imply that heavy alcohol drinking and acute withdrawal do not impact forced swim stress reactions in female C57BL/6J mice. This result was interesting because previous studies on male C57BL/6J mice have found alcohol intake and withdrawal to hinder adaptive learning behavior and cause no change in immobility during the second swim (Hwa et. al., 2019). Due to inherit male and female behavioral differences, perhaps the repeated forced swim test may not be a good measure of alcohol-induced, maladaptive effects in females. Further experimentation of the FST, along with other stress tests, with a larger cohort size of female C57BL/6J mice would elucidate a better explanation for these results.

TMT was used in the exposure to predator odor tests as a fear-inducing stimulus because it reliably causes stress adaptive reactions, even in laboratory bred mice (Fendt & Endres, 2008). Unlike other predator odors which are variable by diet, sex, and environment, TMT is a single molecule that has consistent properties, allowing for reproducibility of experiments (Fendt & Endres, 2008). Mice are first exposed to a baseline control of just the object with a clean cotton swab in their cage, before the addition of TMT. It was found that at baseline, alcohol drinking females showed statistically significant increased defensive burying than water drinking females [Figure 3B]. Defensive burying refers to the displacing of bedding with forepaws or shoveling movements with heads in the presence of aversive stimulation or a threat. Burying behavior has been extensively used as a measure of fear/anxiety in rodent models (Boer & Koolhaas, 2003). Thus, although no real threat was present in the baseline trial, the extensive burying behavior in alcohol drinking female mice signifies that there likely exists a higher baseline level of anxiety just from alcohol dependence and protracted withdrawal in these mice.

Previous studies on C57BL/6J male mice have shown that norBNI increases defensive burying and avoidance of TMT compared to the alcohol control, which exhibits maladaptive stress behaviors (Hwa et. al. 2019). Similarly, this study shows that in female mice, norBNI rescued the alcohol-induced impairment in regards to defensive burying behavior [Figure 4C], but did not have any significant effect on time spent in contact with the object [Figure 4A] or in the far corners of the cage [Figure 4B]. It is also important to note that, in females, norBNI increases burying behavior in water drinkers as well. This suggests that systemic blockade of KORs throughout the entire nervous system leads to a non-specific increase in burying behavior in female mice. This is in contrast to previous published reports in male rats showing that the KOR antagonist DIPPA reduced defensive burying of a shock probe (Carr and Lucki, 2012). It is possible that site-specific infusion of norBNI, for example into the bed nucleus of the stria terminalis (BNST), could yield different results only in the alcohol drinkers.

There was no difference in the TMT contact and time spent in the far corners between alcohol drinking and water drinking control females. This was also unlike previous data collected from males, which exhibit alcohol-induced deficits in avoiding the predator odor. Although the inconsistency in the behaviors between sexes could have been a consequence of this study's small sample size, alcohol and withdrawal could cause female mice to fundamentally cope differently with stressors than they would in male mice. Some of these differences could be due to maternal instincts such as nest building that could contribute to increased burying behavior. After additional experimentation, if a large sexual dimorphism in stress coping mechanisms still exists, hormonal differences between sexes, and their interactions with the nervous system, could be responsible for the observed results, and should be further investigated.

We observed and quantified behaviors in the presence of peanut oil to determine whether alcohol-induced olfaction deficits were responsible for any differences between alcohol and water drinking animals, in the predator odor response. Peanut oil was used because it is an appetitive scent for mice (Rodriguez-Romaguera et al., 2020OURCE?). It was found that olfaction was unaffected by alcohol consumption because alcohol drinking mice exhibited the same behaviors in the presence of peanut oil as water drinking controls. However, this experiment was conducted using male C57BL/6J mice. Since we observed many sex differences in stress test behaviors, future studies should investigate peanut oil response in alcohol-dependent females. An alcohol-induced, aversive female response to the peanut oil, similar to that of the baseline trial of the exposure to predator odor test, may lead to a better understanding of female mice instincts and behavior after alcohol exposure and withdrawal.

In conclusion, this study found that female C57BL/6J mice with intermittent access to alcohol over a period of six weeks will increase their preference for alcohol over water to an average of about 60% and will also increase their alcohol intake per unit body weight during drinking sessions. This data is consistent with male C57BL/6J mice; however, female mice intake a larger volume of alcohol per body weight than do male mice. Compared to previous data found for the FST and predator odor test in male C57BL/6J mice, it seems that female mice may show different stress coping behaviors and a different baseline level of anxiety after alcohol drinking and withdrawal. Thus, norBNI also had different effects on the two sexes. This could be due to a wide variety of factors such as the sex-specific differences in alcohol intake volume, hormones, or stress responses. The implication of these results suggests that male and female people with alcohol use disorders may require different behavioral or pharmacological

treatments, as there may be underlying factors unique to each sex that contribute to addiction, anxiety, and relapse.

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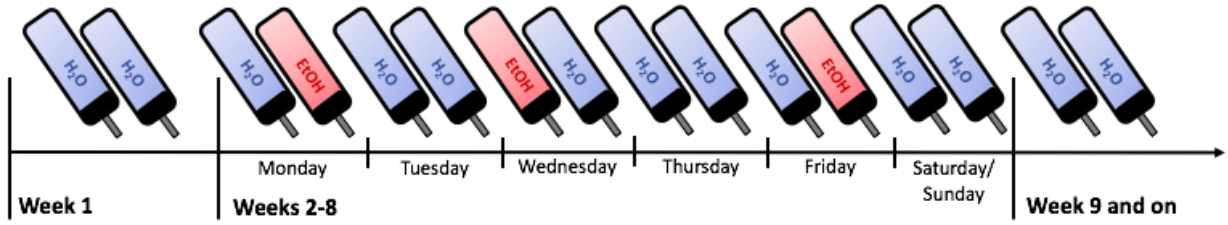
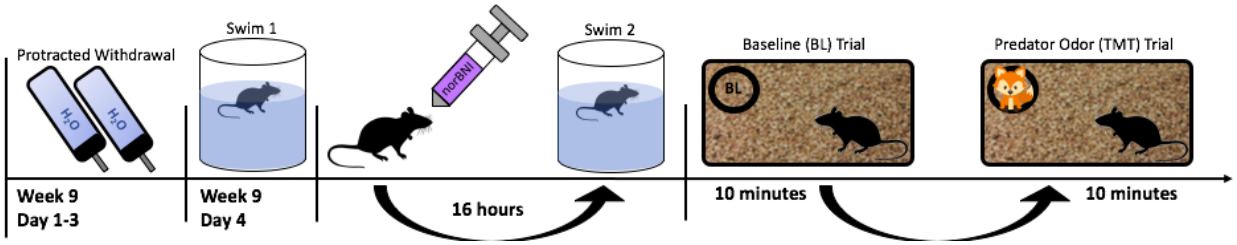
SUPPLEMENTAL FIGURES



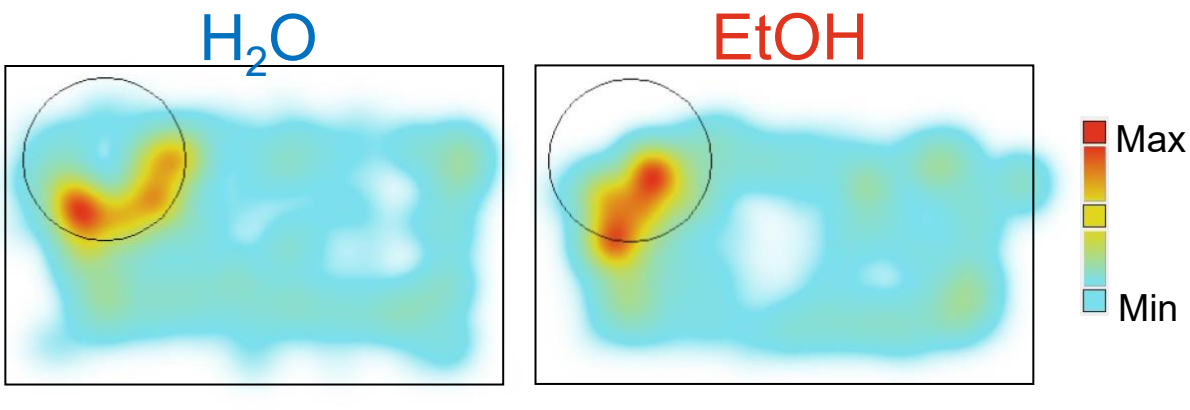
Supplemental Figure 1: Intermittent Access (IA) to Ethanol Schedule.

Ethanol exposure began the second week after mice had acclimated to single housing and two bottle system. Ethanol was available for the IA mice Monday, Wednesday, and Friday and rotated between right and left sides. Mice had a total of 18 drinking sessions over 6 weeks, each 24 hours long.



Supplemental Figure 2: Behavior Testing Schedule

Mice underwent 3 days of protracted withdrawal before the first trial of the forced swim test. The second forced swim test occurred 72 hours after the first. Mice were injected with either norBNI or saline 16 hours prior to the second trial. Then, the mice ran a baseline trial for exposure to predator odor test, where they were exposed to an unscented cotton swab object in their home cage for 10 minutes. Immediately after that, TMT was applied to the cotton swab and the mouse was observed for 10 additional minutes.



Supplemental Figure 3: Heat Maps of Spatial Location During Peanut Oil Experiment:

The circle represents the peanut oil object. Red indicates maximum time spent in that location, while blue indicates minimum time spent. Alcohol history did not have an effect on time in contact with the peanut oil suggesting no alcohol-related deficits in olfaction.