Drinking in the Dark: Voluntary Co-consumption of Nicotine and Alcohol for Binge-like Drinking Behavior in Mice

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Drinking in the Dark: Voluntary Co-Consumption of Nicotine and Alcohol for Binge-like Drinking Behavior in Mice

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Katherine Lynne Benson
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COMORBID ADDICTION OF NICOTINE AND ALCOHOL IN MICE

Abstract

Alcohol and nicotine are two of the most commonly abused drugs across the United States. Given the high rates of comorbidity, it remains a pressing public health concern to determine how the two drugs interact within the CNS, and how this impacts addictive behavior. The present study investigated the effect of comorbid abuse of nicotine and alcohol on stress and anxiety-like withdrawal symptoms, as both are associated with increased rates of relapse. A voluntary co-consumption, two-bottle choice paradigm was used with nicotine and alcohol to induce binge-like drinking behavior in mice following the drinking in the dark (DID) model of consumption. One bottle of water and one experimental bottle that contained nicotine (5-30 μg/ml), alcohol (3-20% v/v), nicotine+alcohol, or water were placed in each cage. Adult C57BL/6J mice (20 male, 20 female) mice had access to the substance of interest for 4 hours within their dark, or active, period in a 24 hour cycle. Plasma corticosterone levels were measured at baseline (before any substances were introduced) and again in the acute withdrawal period in n=16 mice. The marble burying task was done as a behavioral measure of anxiety-like withdrawal symptoms in n=24 mice. I expected to see an increase in stress and anxiety-like withdrawal symptoms in the co-morbid condition compared to the alcohol and nicotine conditions alone, and all drug conditions compared to the water control condition. I did not find significant results or trends for corticosterone or the MBT. I saw a trend towards significance in the main effect of sex on anxiety-like withdrawal symptoms. The present study contributes to the knowledge surrounding comorbid alcohol and nicotine dependence in hopes to better understand this addiction.
COMORBID ADDICTION OF NICOTINE AND ALCOHOL IN MICE

Introduction

Alcohol and nicotine are drugs widely abused both independently and codependently across the United States. Tobacco, of which the active component is nicotine, is the number one preventable cause of death in the U.S. as of 2016, and alcohol is the third leading cause of preventable death in the U.S. as of 2015 (Tips from Former Smokers, 2018; Alcohol Facts and Statistics, 2015). As of 2016, approximately 15.5% of U.S. adults 18 year and older were chronic cigarette smokers (Tips from Former Smokers, 2018). In addition, about 10.7% of young adults aged 18-25 years old, and 5.2% of adults 25 and older suffer from Alcohol Use Disorder, a disorder of alcohol addiction (Ahrnsbrak et al., 2016). Beyond their independent prevalence, previous studies found an estimated 80-90% of alcoholics are smokers and an estimated 90% of smokers are regular drinkers (Keenan et al., 1990; Miller & Gold, 1998). These high rates of comorbid abuse have severe implications for addiction treatment and recovery. People who simultaneously abuse nicotine and alcohol have higher rates of relapse for both drugs (McKee & Weinberger, 2013). Given the high rates of comorbid abuse it remains an important topic of research to determine the nature of the relationship between the addictive properties of the two substances. It is unknown exactly what drives the high rate of comorbidity; whether smoking tobacco cigarettes increases the urge to drink alcohol, drinking alcohol increases the urge to smoke, or both.

Alcohol is a “promiscuous” drug, meaning it has many sites of action within the brain and body to induce its effects. It acts primarily on ion channels and receptors of neurons to alter brain function. Alcohol alters various neurotransmitter receptor subtypes,
mostly by acting to inhibit receptors for the primary excitatory neurotransmitter glutamate (in particular the NMDA and AMPA subtypes) and by activating receptors for the primary inhibitory neurotransmitter GABA, but also through the potentiation of nicotinic acetylcholine and serotonergic receptors (Vengeliene et al., 2008). Additionally, acute alcohol also induces changes in dihydropyridine-sensitive L-type voltage-gated calcium and G-protein activated inwardly rectifying K+ channels (Vengeliene et al., 2008). The action of alcohol at these sites creates a cascade of signaling within the brain that can further stimulate the release of neurotransmitters such as dopamine (DA), glutamate, and noradrenaline (Vengeliene et al., 2008). The alteration in neurotransmitter signaling initiated by alcohol produces rewarding effects via the mesolimbic DAergic pathway, projecting from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) and the prefrontal cortex (Vengeliene et al., 2008).

Nicotine works as an agonist at nicotinic acetylcholine receptors (nACHRs) found on cholinergic and DAergic neurons to stimulate the release of acetylcholine and DA. nACHRs are pentameric, excitatory receptors, with their channels allowing an influx of Na+ and Ca2+ and efflux of K+. Similar to the effects seen with alcohol, the release of DA in the striatum and VTA is believed to cause the rewarding effects of nicotine. Acting as heteroreceptors, nACHRs on the axon terminals of DAergic neurons in the striatum and NAc bind nicotine and stimulate the release of DA in the reward pathway (Van Skike et al., 2016). Repeated use of nicotine results in an increase in the number of nACHRs present on neurons, specifically receptors containing α7 and α4β2 subunits. However, while there is an increase in the number of these receptors, they are less sensitive to the
binding of nicotine, which in turn causes a decrease in excitation and subsequent release of DA. This decrease in DA is thought to lead not only to a lack of reward, but also to the unpleasant withdrawal symptoms associated with nicotine addiction such as anxiety and irritability (Van Skike et al., 2016).

The most prominent mechanistic overlap between alcohol and nicotine, and a primary area of interest for researchers, is nAChRs. The reinforcing effects of both drugs are thought to be partially mediated through actions at nAChRs on DAergic neurons in the VTA which extend to the mesolimbic system or the primary reward system, including the NAc (Van Skike et al., 2016). Previous research has found simultaneous administration of alcohol and nicotine has an additive effect on DA levels, whereas when nicotine is administered before alcohol the elevation in DA levels is reduced (Schlaepfer, Hoft, & Ehringer, 2008; Van Skike et al., 2016). While the nature of this relationship is still not clear, this shows a definite biochemical interaction between the rewarding effects of nicotine and alcohol with comorbid abuse.

Nicotine and alcohol also overlap in their effects on cortisol, or corticosterone (the rodent equivalent), via the hypothalamic-pituitary-adrenal axis (HPA axis). The HPA-axis is a neuroendocrine signaling pathway in which the paraventricular nucleus of the hypothalamus releases the hormones corticotropin-releasing factor (CRF) and/or arginine-vasopressin into blood vessels connected to the pituitary gland (Stephens & Wand, 2012). Upon receiving the signal, the pituitary gland secretes the adrenocorticotropic hormone which signals for the adrenal glands to release glucocorticoids, the primary one in humans being cortisol and in rodents being
corticosterone (Stephens & Wand, 2012). Cortisol and corticosterone elicit many of the stress responses observed following simulation of the HPA-axis and negatively regulate hypothalamic release of the CRF and arginine-vasopressin to maintain homeostatic hormone levels (Stephens & Wand, 2012). Nicotine and alcohol consumption independently elevate cortisol and corticosterone levels by stimulating the HPA-axis, though the exact mechanisms by which both drugs interact with the HPA-axis are not completely understood (Lovallo, 2006). Uncovering the nature of these interactions is critical, as malfunction of the HPA-axis following the onset of addiction and subsequent increases in cortisol levels are associated higher rates of relapse for both drugs (Stephens & Wand, 2012; Tweed et al., 2012). Additionally, elevations in corticosterone levels positively correlate to anxiety-like withdrawal systems in studies using both nicotine and alcohol. Such symptoms can be measured in a number of behavioral paradigms, including the marble burying task, the elevated plus task, and light-dark exploration (Norman et al., 2015; George et al., 2007). In addition to their combined effects on the reward pathway, the combined interactions of nicotine and alcohol with the HPA-axis may be a key component to understanding why rates of stress-induced relapse are increased for those struggling with a comorbid addiction of these drugs.

Past research using animal models with voluntary consumption paradigms of both alcohol and nicotine has been limited. Most voluntary consumption experiments have been done with either nicotine or alcohol, but not both. Studies of comorbid abuse have largely had one drug voluntarily consumed, while the other was administered by an experimenter. This limits the application of such studies to humans given that with human
comorbid addiction, both drugs are voluntarily consumed. Voluntary co-consumption models with both alcohol and nicotine more closely resemble the drug abuse behavior of humans, yet research done with voluntary co-consumption is sparse. Rourke et al. (2016) utilized a voluntary co-consumption model with both alcohol and nicotine in a three bottle paradigm that presented water, a nicotine solution, and an alcohol solution in three separate bottles. This limits what can be learned about the nature of drug interaction between nicotine and alcohol because it does not control for the time or the quantities that the two drugs are being consumed in relation to one another. The current study addressed this with a two bottle paradigm in which there are three experimental groups, a nicotine group, an alcohol group and a co-consumption group, strictly regulating the three kinds of consumption.

In the drinking in the dark (DID) model of consumption, rodents have access to the substance of interest for a limited number of hours within their dark, or active, period in a 24 hour cycle. This drinking model is thought to increase consumption of the drug of interest over a shortened period of time by putting the mouse through withdrawal from the drug every 20 hours (Holgate et al., 2017). This has proven to be an effective model for the binge drinking behavior seen in humans, and has reliably gotten mice to consume alcohol to the point of intoxication (Holgate et al., 2017; Rhodes et al., 2005; Ryabinin et al., 2003). Although the DID paradigm reliably results in binge-like blood alcohol concentrations (Rhodes et al. 2005; Rhodes et al. 2007), few studies have investigated the effects of alcohol withdrawal using a DID model and those that did found conflicting results (Crabbe et al., 2012; Lee et al., 2018). A recent study has shown that DID is also
successful in increasing voluntary nicotine consumption in mice (Kasten, Frazee, & Boehm, 2016), though no study to date has looked at withdrawal following DID access to nicotine. The elevated levels of drug consumption seen with the DID model more closely resemble the binge drinking behavior seen in humans, thus further implicating DID as a good model for studying comorbid addiction of nicotine and alcohol in mice. To our knowledge, there is no published research investigating comorbid addiction of nicotine and alcohol using this binge drinking model.

Given the high rates of comorbid addiction to nicotine and alcohol, it remains a pressing public health concern to determine how the two drugs interact within the central nervous system, and how this, in turn, impacts addictive behavior. Animal paradigms for research remain critically important as models in which the variables of addiction can be experimentally controlled and manipulated. The present study sought to add to the growing base of knowledge on this subject. I used a voluntary co-consumption model with nicotine and alcohol to induce binge-like drinking behavior in mice and measured the subsequent impact on withdrawal stress using a corticosterone assay and a marble burying behavioral assay. I used the C57BL/6J genetic strain of mice, as they are widely used in the field of addiction research and have been long established to have a predisposition to high ethanol consumption (Rogers, 1966). I hypothesized that the co-consumption of nicotine and alcohol has an additive effect on the activation of the HPA-axis and subsequent corticosterone signaling and stress responses. Accordingly, I expected to see elevated corticosterone levels in our nicotine+alcohol conditions compared to either nicotine or alcohol alone, and all of the experimental conditions
should be elevated compared to the water control. In our marble burying assay I also expected to see more marbles buried as an indication of increased anxiety-like withdrawal symptoms in our nicotine+alcohol conditions compared to either nicotine or alcohol alone, and that all experimental conditions should bury more marbles than the water only control condition.

Method

Subjects

Jackson Laboratory (Bar Harbor, ME) provided both male and female mice of the C57BL/6J genetic strain (n=20 males, 20 females) and all mice were at least 8 weeks of age at the outset of the experiment. Mice were housed individually in a 12 hour reverse light-dark cycle. Food and water were available ad libitum to all mice for the duration of the experiment. Mouse weight was documented on a weekly basis. All animal care was done in accordance with the National Institutes of Health Office of Laboratory Animal Welfare and the Institutional Animal Care and Use Committee at Butler University protocol.

Drugs and Chemicals

All chemicals for the experiment, including 190 proof alcohol and nicotine hydrogen tartrate salt were purchased from Sigma (St. Louis, MO). Tap water was used to mix all concentrations of solutions. Nicotine concentrations were not pH adjusted or sweetened and were reported as free base.

Corticosterone Measurements
A random subgroup of mice (n=8 males, n=8 females) from the colony was chosen prior to the experiment and their blood (approximately 100 µl) drawn to extract plasma samples for the corticosterone assays. A baseline corticosterone measurement was taken before drug introduction and a measurement was taken again 24 hours into drug withdrawal. Corticosterone levels were analyzed using the corticosterone enzyme-linked immunosorbent (ELISA) assay from Immunodiagnostic systems (Gaithersburg, MD).

**Drinking Model**

As prescribed by the DID paradigm, mice had access to water in a bottle and food *ad libitum* 24 hours a day. Mice were presented with a second bottle containing alcohol (3-20% v/v) and/or nicotine (5-30 µg/ml) for 4 hours each day in excess. The mice were housed on a 12:12 hour reverse light dark cycle and drug access period began approximately 3 hours into their dark, or active, cycle. The mice had access to the alcohol (3-20% v/v) and/or nicotine (5-50 µg/ml) for no more than 4 hours in a 24 hour period. This paradigm was repeated in a 24 hour cycle, Monday-Friday, for 4 weeks.

**Anxiety-like Behavior**

As a behavioral measure of withdrawal-induced anxiety, the marble burying task (MBT) was performed with a subgroup of mice (n=12 males, n=12 females) approximately 24 hours into the drug cessation period, during the acute withdrawal period. There was no overlap between the sample of mice used for this measure and the mice used for the corticosterone assay. The MBT is a well supported measure of withdrawal-induced anxiety behavior (Zhao-Shea et al., 2013; Zhao-Shea et al., 2015). Mice were habituated to the task cage for 2 days prior to the task, spending at least 30
minutes a day in the task cage. To perform the experiment, black, opaque glass marbles with a 1.5 cm diameter were sterilized and placed on 5-6 cm of bedding, 4 cm apart, in 5 rows of 4, with a total of 20 marbles. Each mouse was placed in the test cage and left undisturbed for 30 minutes and then returned to their home cage. Marbles buried to at least ⅔ their depth were scored as “buried” by three independent observers blinded to the condition of each animal, with the average number of marbles buried serving as the experimental measure for the task where more marbles buried corresponds to higher anxiety.

Statistical Analysis

Evaporation and leakage in the bottles was controlled for in the data by subtracting control bottle values from the experimental data collected. Control bottles were treated exactly the same as experimental bottles, only their cage had no mice. The control-adjusted data were the raw data. Consumption values were determined by transforming the raw data into of percent alcohol or nicotine concentration, density of alcohol, and the weight of the mouse. Teess transformed values were averaged over the period for each concentration to yield an average daily consumption value for alcohol (g/kg/day) and nicotine (mg/kg/day). Percent preference for nicotine or alcohol was determined with the following equation, (ml of nicotine or alcohol consumed/ total ml of fluid consumed) *100%. A 2 (male and female, between subjects) x 4 (concentration, within subjects) mixed factor ANOVA was used to evaluate consumption and preference data. Additionally, a 2 (sex) x 4 (only nicotine, only alcohol, nicotine+alcohol, water)
between subjects ANOVA was used to evaluate marble burying data. All data were analyzed using the statistical software GraphPad version 8.0.

**Results**

**Alcohol**

*Consumption*

The average voluntary consumption of alcohol (g/kg/4hr) in the alcohol only condition was measured across four weeks at 3, 6, 10, and 20% v/v (weeks 2-4 at 20%) in male and female mice (Fig. 1A). A 2-way mixed factor ANOVA revealed no significant interaction of sex and alcohol concentration. There was a significant main effect of concentration \((F(5, 40)=29.13, p<0.0001)\) and sex \((F(1,8)=9.394, p=0.0155)\). Females consumed more alcohol than males at all concentrations greater than 3% v/v (Fig. 1A), reaching an average of approximately 10 g/kg/4 hr during weeks 2-4. Male consumption was highest in week 4 at 20% v/v (reaching an average of approximately 7 g/kg/4 hr), whereas female consumption was highest in week three at 20% v/v (Fig. 1A).

*Preference*

The average preference for alcohol (g/kg/4hr) in the alcohol only condition was measured across four weeks at 3, 6, 10, and 20% v/v (weeks 2-4 at 20%) in male and female mice (Fig. 1B). A 2-way mixed factor ANOVA revealed no significant interaction of sex and alcohol concentration. There was a significant main effect of concentration \((F(5,40)=7.834, p<0.0001)\), but no significant main effect of sex. For both males and females, alcohol preference peaked at the 10% concentration with a 90% preference.
score, indicating that nearly every animal strongly preferred the alcohol bottle compared to water. This value remained fairly stable at the 20% v/v concentration for the remaining 3 weeks of the experiment (Fig. 1B).

Nicotine

Consumption

The average voluntary consumption of nicotine (mg/kg/4hr) in the nicotine only condition was measured across four weeks at 5, 10, 15, and 30 µg/ml (weeks 2-4 at 30 µg/ml) in male and female mice (Fig. 2A). A 2-way mixed factor ANOVA revealed no significant interaction of sex and nicotine concentration, nor was there a significant sex difference. There was a significant main effect of concentration ($F(5,40)=11.64, p<0.001$). Nicotine consumption rose steadily during the first week and remained steady throughout weeks 2-4 around 1 mg/kg/4 hr. (Fig.2A).

Preference

The average preference for nicotine (mg/kg/4hr) in the nicotine only condition was measured across four weeks at 5, 10, 15, and 30 µg/ml nicotine (weeks 2-4 at 30 µg/ml) in male and female mice (Fig. 2B). A 2-way mixed factor ANOVA revealed no significant interaction of sex and nicotine concentration. There was no significant main effect of concentration or sex. Female nicotine preference was lowest at 5 ug/ml and highest in week 4 at 30 ug/ml (Fig.2B). Male preference leveled out at 10 ug/ml and was highest at 5 ug/ml (Fig. 2B).
Alcohol + Nicotine

Consumption

The average voluntary consumption of alcohol+nicotine (g/kg/4hr and mg/kg/4hr, respectively) in the co-consumption condition was measured across four weeks at 5, 10, 15, and 30 µg/ml nicotine (weeks 2-4 at 30 µg/ml) and 3, 6, 10, and 20% v/v alcohol (weeks 2-4 at 20%) in male and female mice (Fig. 3A, 3B). The results from mice consuming combined alcohol+nicotine can be found in Figure 3, with alcohol consumption on the left y-axis and nicotine consumption on the right y-axis. For males, a 2-way repeated measures ANOVA of concentration and substance (where substance is either alcohol or nicotine) revealed a significant interaction of concentration and substance ($F(5,20)=13.16, p<0.001$) in addition to significant main effects for both concentration ($F(5,20)=12.74, p<0.001$) and substance ($F(1,4)=46.25, p<0.005$). Male mice consumed significantly more alcohol when presented with 20% v/v alcohol + 30 µg/ml nicotine compared to when presented with 3% v/v alcohol + 5 µg/ml nicotine or 6% v/v alcohol + 10 µg/ml nicotine ($p<0.01$). In males, consumption stabilized at 10% v/v alcohol + 15 µg/ml nicotine (Fig. 3A).

For females, a 2-way repeated measures ANOVA of concentration and substance revealed a significant interaction of concentration and substance ($F(5,20)=4.208, p<0.01$) in addition to significant main effects for both concentration ($F(5,20)=3.797, p<0.05$) and substance ($F(1,4)=219.9, p<0.001$). Similar to what was seen in males, female mice consumed significantly more alcohol when presented with 20% v/v alcohol + 30 µg/ml nicotine compared to when presented with 3% v/v alcohol + 5 µg/ml nicotine or 6% v/v alcohol + 10 µg/ml nicotine ($p<0.01$). In females, consumption stabilized at 10% v/v alcohol + 15 µg/ml nicotine (Fig. 3B).
nicotine compared to when presented with 3% v/v alcohol + 5 µg/ml nicotine or 6% v/v alcohol + 10 µg/ml nicotine (p<0.01). In females, consumption stabilized in week 2 at 20% v/v alcohol + 30 ug/ml nicotine on and was highest in the third week at 20% v/v alcohol + 30 ug/ml nicotine concentrations (Fig. 3B).

Preference

The average preference for alcohol+nicotine (g/kg/4hr and mg/kg/4hr, respectively) in the co-consumption condition was measured across four weeks at 5, 10, 15, and 30 µg/ml nicotine (weeks 2-4 at 30 µg/ml) and 3, 6, 10, and 20% v/v alcohol (weeks 2-4 at 20%) in male and female mice (Fig. 3C). A 2-way ANOVA of concentration and substance (either alcohol or nicotine) revealed no significant interactions or main effects. Preference for both males and females was highest at the 10% v/v alcohol + 15 ug/ml nicotine concentrations (Fig. 3C).

Comparison

Alcohol vs Alcohol + Nicotine

The average alcohol consumption and preference in both alcohol alone and alcohol+nicotine mice was compared across four alcohol concentrations (3-20% v/v) in both male and female mice (Fig. 4). In male mice, a 2-way mixed factor ANOVA revealed no significant interaction between concentration and group (alcohol vs alcohol+nicotine), nor was there a significant main effect of group. There was a significant main effect of concentration ($F(5, 40) = 34.71, p<0.0001$) on alcohol
consumption, where consumption rose as the concentration was increased across days (Fig. 4A). A similar pattern was found for alcohol preference in male mice exposed to either alcohol alone or alcohol+nicotine (Fig. 4B).

In female mice, a 2-way mixed factor ANOVA revealed no significant interaction between concentration and group (alcohol vs alcohol+nicotine), nor was there a significant main effect of group. There was a significant main effect of concentration ($F(5, 48) = 15.45$, $p<0.0001$), where consumption rose as the concentration was increased across days (Fig. 4A). A similar pattern was found for alcohol preference in female mice exposed to either alcohol alone or alcohol+nicotine (Fig. 4B). Females showed greater, though non-significant, consumption compared to males in both alcohol and alcohol + nicotine conditions (Fig. 4A). Additionally, alcohol and alcohol + nicotine preference increased as concentration increased (Fig. 4B).

**Nicotine vs Alcohol + Nicotine**

The average nicotine consumption and preference in both nicotine alone and alcohol+nicotine mice was compared across four nicotine concentrations (5-20 \(\mu g/ml\)) in both male and female mice (Fig. 5). In male mice, a 2-way mixed factor ANOVA revealed no significant interaction between concentration and group (nicotine vs alcohol+nicotine). There was a main effect of concentration ($F(5, 48) = 9.880$, $p<0.0001$) and group ($F(1, 48) = 12.20$, $p=0.001$). Overall, as the concentration was increased, consumption of nicotine in male mice also increased (Fig. 5A). For the group factor, collapsed across concentration, alcohol+nicotine males consumed significantly more
nicotine compared to nicotine alone male (Fig. 5A). A similar pattern was found for nicotine preference in male mice exposed to either nicotine alone or alcohol+nicotine (Fig. 5B), where alcohol+nicotine mice had a stronger preference for nicotine compared to nicotine alone mice.

For females, a 2-way mixed factor ANOVA revealed no significant interaction between concentration and group (nicotine vs alcohol+nicotine). Similar to the results observed in male mice, there was a main effect of concentration ($F(5, 48) = 8.259, p<0.0001$) and group ($F(1, 48) = 22.70, p<0.0001$). Overall, as the concentration was increased, consumption of nicotine in female mice also increased (Fig. 5A). For the group factor, collapsed across concentration, alcohol+nicotine females consumed significantly more nicotine compared to nicotine alone females. A similar pattern was found for nicotine preference in female mice exposed to either nicotine alone or alcohol+nicotine (Fig. 5B), where alcohol+nicotine mice had a stronger preference for nicotine compared to nicotine alone mice.

Alcohol + nicotine consumption was greater than nicotine consumption across all concentrations exclusive of 5 ug/ml nicotine (Fig. 5A). While not tested for significance, alcohol+nicotine preference was greater than nicotine preference across all concentrations for both males and females, exclusive of males at 5 ug/ml nicotine (Fig. 5B).

**Corticosterone Levels**
Levels of the stress hormone corticosterone were examined in a subset of mice prior to the start of the experiment (basal) and 24 hours after alcohol and/or nicotine was removed (withdrawal). A 3-way between subjects ANOVA revealed no significant interactions nor any significant main effects of sex, group, or time (basal or withdrawal). A total of four corticosterone samples from the withdrawal day were excluded from analysis due to instrumentation error. Although not statistically significant, as shown in Figure 6, male mice had lower basal corticosterone levels compared to their female counterparts across all conditions. This sex difference is typical for this assay (Rhodes & Rubin, 1999). The opposite was true during alcohol and/or nicotine withdrawal, with male mice exhibiting higher corticosterone levels compared to female mice across all conditions, exclusive of water only. Male corticosterone levels were higher in withdrawal for the nicotine only and alcohol only conditions compared to basal levels (Fig. 6). Female corticosterone levels were lower in withdrawal across all conditions compared to basal levels (Fig. 6).

**Marble Burying Task**

During acute withdrawal from alcohol and/or nicotine (approximately 24 hours after cessation), a subset of mice underwent the marble burying task in order to measure withdrawal-induced anxiety-like behavior. A 2-way between subjects ANOVA revealed no significant interaction between sex and condition on marbles buried. There were also no main effects of sex or condition on marbles buried, though there was a trend towards significance for sex ($F(1, 16)=2.055, p=0.17$).
**Discussion**

The purpose of our study was to better understand the relationship between the co-morbid abuse of alcohol+nicotine and the HPA-axis mediated stress response during withdrawal. I sought to do this with a mouse model using the DID paradigm and using the MBT and corticosterone levels to measure stress in withdrawal. I hypothesized that the co-consumption of nicotine and alcohol has an additive effect on HPA-axis activation and subsequent corticosterone signaling and stress responses, and therefore predicted that mice in the co-consumption condition would have elevated corticosterone levels and anxiety-like withdrawal symptoms compared to either nicotine or alcohol alone. However, our results did not support this hypothesis or predictions. I found no significant changes in corticosterone levels during withdrawal compared to basal levels in any condition (Fig. 6). I also found no significant difference in the number of marbles buried between conditions in the MBT. (Fig. 7).

Though our hypothesis was not supported, our findings do support previous research done by our lab using a continuous access two-bottle paradigm. Our prior research found that the presence of alcohol affects nicotine consumption, but the presence of nicotine does not affect alcohol consumption. This trend is also evident in the results of the present study when comparing the consumption of nicotine or alcohol alone to the co-consumption condition. Females consumed significantly more in the alcohol+nicotine condition compared to the nicotine alone condition (Fig. 3a). This result was replicated in males (Fig. 3b). Neither males nor females showed a difference in consumption when
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comparing the alcohol+nicotine condition to the alcohol alone condition. Our concurrent findings suggest alcohol consumption may increase nicotine consumption in mice, but not vice-versa.

While no significant increases in co-consumption were observed, it appears as though the DID paradigm was effective in inducing binge-like drinking behavior. Mouse consumption levels in the alcohol+nicotine co-consumption condition in the current study were in line with levels of that seen in Kasten, Frazee, & Boehm, 2016’s single bottle co-consumption DID paradigm with C57BL/6J mice in which saccharin was mixed with nicotine to promote drinking. Ethanol consumption (g/kg/4hr) in the ethanol only condition also replicated ethanol consumption levels found by Rhodes et al. 2005 using a DID paradigm with C57BL/6J mice. Additionally, while not tested for significance, comparison to prior unpublished results from our lab using a 2-bottle continuous access paradigm shows our DID paradigm induced higher levels of nicotine, alcohol, and alcohol+nicotine consumption both in male and female mice. To our knowledge, this is the first study showing a two-bottle DID paradigm is an effective model for binge-like co-consumption of nicotine and alcohol in C57BL/6J mice.

The non-significant results for corticosterone levels observed in the current study may in part be explained by a study done by Richardson et al., 2009. They found that HPA-axis response and subsequent corticosterone levels decreased in alcohol-dependent mice compared to a low-consumption group of mice. Another study found decreased corticosterone levels in mice following chronic alcohol abuse during withdrawal (Rassmusen et al., 2000). The results of a study on repeated alcohol injections and
withdrawal effects on the HPA-axis in rats also showed no change in corticosterone levels from basal to withdrawal (Flemming et al., 2019). This could potentially explain why in the alcohol only and the co-consumption conditions corticosterone levels either remained the same or decreased during withdrawal, and all changes were non-significant. On the contrary, Olive et al., 2002 found binge drinking alcohol increased corticotropin releasing factor (CRF) (a precursor to corticosterone) levels in withdrawal and that subsequent drinking decreased CRF levels in mice; however the duration of the study was only two weeks compared to four weeks in the current study and the aforementioned studies. As for nicotine, prior research has shown acute and chronic nicotine administration via injections elevates corticosterone levels in mice during administration (Cam & Bassett, 1983; Pauly, Grun, & Collins, 1992). One study looking at nicotine withdrawal found that chronic nicotine injections in rats elevated plasma corticosterone levels during the withdrawal period (Benwell & Balfour, 1983). The nicotine levels reached with voluntary consumption in the present experiment may not have been high enough to replicate this result. It also may be that the route of administration differentially impacts nicotine’s interaction with the HPA-axis. Taken together, the results of the current study along with prior research suggest that there are elements of alcohol, nicotine, and the HPA-axis interactions that are still not completely understood, particularly concerning chronic voluntary consumption.

Unfortunately, due to instrumentation error that occurred during the corticosterone assay, I had to exclude four of the 16 plasma samples taken during withdrawal, which severely lessened the statistical power and our ability to find any significant results.
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Additionally, several plasma samples included were “muddied” or red, meaning the plasma was not fully separated from the cell matter, potentially interfering with the results of the assay. Both the reduced number and quality of samples for the corticosterone assay may explain the current aberrant results for the assay. However, if the non-significant results for the corticosterone assay were due solely to methodological error, one would expect the MBT results to still reflect a heightened anxiety in withdrawal for the experimental conditions compared to the water control condition. Contrary to this, the MBT results also showed no significant differences between conditions. Prior research on the MBT shows that it reflects increased anxiety-like withdrawal symptoms in mice in alcohol withdrawal; however, results for nicotine withdrawal are mixed (Perez & Biasi, 2015; Turner, Castellano, & Blendy, 2011; Zhoa et al., 2015). A methodological study found that MBT results did not correlate with other behavioral measures of anxiety, suggesting it may not be the most accurate method to measure anxiety-like withdrawal symptoms and offering a possible explanation for the observed results (Thomas et al., 2009).

Given some of the limitations of the current study discussed above, there are many possibilities for future experiments. Future studies should improve methodology by increasing the sample size for the corticosterone assay to n=20 (10 male, 10 female) to account for plasma collection errors in the minimum number of mice needed to achieve sufficient statistical power when analyzing results. Future studies may also try using other behavioral measures of anxiety-like withdrawal symptoms, or other methods in concurrence with the MBT, such as light-dark exploration or somatic sign observation, to
see if the MBT may be ineffective for measuring anxiety-like withdrawal symptoms in a comorbid paradigm with nicotine and alcohol. As the current study established that the DID model is effective in inducing binge-drinking behavior for nicotine and alcohol in a co-consumption paradigm, future studies may run a 2 phase experiment in which a DID model is used to induce binge drinking, followed by a period of continuous access to the substance of interest. This may help to uncover how repeated binge drinking episodes affect long term substance abuse in nicotine and alcohol. Future studies may also look to other substances to investigate comorbid addiction. Studies of alcohol and caffeine may provide insight into the effects of popular caffeinated drinks consumed with alcohol. As recreational marijuana becomes more widespread with legalization, it will be important for future research to examine its comorbid effects with both nicotine and alcohol.

The current study sought to add to the growing base of knowledge on the comorbid addiction of nicotine and alcohol. The anxiety-like withdrawal symptoms associated with withdrawal are a major hindrance in getting chronic substance abusers to quit and continue to abstain once they have quit (Becker, 2014; Benowitz, 2008). Further, nicotine and alcohol are two of the most widely abused substances in the United States (Tips from Former Smokers, 2018) (Alcohol Facts and Statistics, 2015). A report published in 2011 found excessive alcohol consumption to cost the U.S. economy 223.5 billion dollars annually and according to the CDC, smoking-related illnesses cost the U.S. more than 300 billion dollars annually (Economic Cost, 2011; Economic Trends, 2018). Studying nicotine and alcohol abuse together remains a pressing issue, with estimations up to 90% for their co-morbid abuse (Keenan et al., 1990; Miller & Gold, 1998). The
results of our study may contribute to a better understanding of effective methodology for research on the comorbid addiction of nicotine and alcohol. The current study also adds support to previous findings that alcohol consumption, at least initially, may drive an increase in nicotine consumption although not vice-versa; however, further research must be done before these findings can be confirmed in organisms other than mice.
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Figure 1 Consumption of and preference for alcohol in C57BL/6J mice in a two-bottle choice DID paradigm increases with concentration. The average voluntary consumption of and preference for alcohol in the alcohol only condition was measured across four weeks across at 3, 6, 10, and 20% v/v (weeks 2-4 at 20%) in male and female mice. A) A 2-way mixed factor ANOVA revealed a significant main effect of concentration ($p<0.001$) and sex ($p=0.0155$). B) A 2-way mixed factor ANOVA revealed a significant main effect of concentration ($p<0.001$).
Figure 2 Consumption of nicotine in C57BL/6J mice in a two-bottle choice DID paradigm increases with concentration. The average voluntary consumption of and preference for nicotine in the nicotine only condition was measured across four weeks across at 5, 10, 15, and 30 µg/ml (weeks 2-4 at 30 µg/ml) in male and female mice. A) A 2-way mixed factor ANOVA revealed a main effect of concentration ($p<0.001$). B) A 2-way mixed factor ANOVA revealed no significant main-effects or interactions.
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Figure 3 Consumption of alcohol+nicotine in C57BL/6J mice in a two-bottle choice DID paradigm increases with concentration. The average voluntary consumption of alcohol+nicotine in the co-consumption condition was measured across four weeks across at 5, 10, 15, and 30 µg/ml nicotine (weeks 2-4 at 30 µg/ml) and 3, 6, 10, and 20% v/v alcohol (weeks 2-4 at 20%) in male and female mice. A) A 2-way mixed factor ANOVA revealed a significant interaction of substance and concentration ($p<0.001$) and significant main effects of concentration ($p<0.001$) and substance ($p<0.005$) in male mice. Male mice consumed significantly more at 20% v/v + 30 µg/ml than at 3% v/v + 5 µg/ml or 6% v/v + 10 µg/ml. B) A 2-way mixed factor ANOVA revealed a significant interaction of substance and concentration ($p<0.01$) and significant main effects of concentration ($p<0.05$) and substance ($p<0.001$) in female mice. Female mice consumed significantly more at 20% v/v + 30 µg/ml than at 3% v/v + 5 µg/ml or 6% v/v + 10
µg/ml. C) A 2-way ANOVA of concentration and substance revealed no significant interactions or main effects for alcohol preference.

Figure 4 Consumption of and preference for alcohol is similar in alcohol only and alcohol+nicotine conditions in C57Bl/6J mice. The average alcohol consumption and preference in both alcohol alone and alcohol+nicotine mice was compared across four alcohol concentrations (3-20% v/v) in both male and female mice. A) In male mice, a 2-way mixed factor ANOVA revealed a significant main effect of concentration on alcohol consumption ($p<0.0001$). In female mice, a 2-way mixed factor ANOVA also
revealed a significant main effect of concentration ($p<0.0001$). B) Male and female preference for alcohol in both conditions followed the same pattern as consumption.

Figure 5 Consumption of and preference for nicotine is greater with combined alcohol+nicotine consumption compared to nicotine only. The average nicotine consumption and preference in both nicotine alone and alcohol+nicotine mice was compared across four nicotine concentrations (5-20 $\mu$g/ml) in both male and female mice. A) In male mice, a 2-way mixed factor ANOVA revealed main effects of concentration ($p<0.0001$) and group ($p=0.001$). For females, a 2-way mixed factor ANOVA revealed main effects of concentration ($p<0.0001$) and group ($p<0.0001$). B) Male and female preference for nicotine in both conditions followed the same pattern as consumption such
that preference was higher in the alcohol+nictine condition than the nicotine only condition.

Figure 6 Basal and withdrawal corticosterone levels did not differ significantly in C57Bl/6J mice. Levels of the stress hormone corticosterone were examined using an ELISA corticosterone assay in a subset of mice prior to the start of the experiment (basal) and 24 hours after alcohol and/or nicotine was removed (withdrawal). A 3-way between subjects ANOVA revealed no significant interactions nor any significant main effects of sex, group, or time (basal or withdrawal). A total of four corticosterone samples from the withdrawal day were excluded from analysis due to instrumentation error.
Figure 7 Control and experimental conditions did not significantly differ in anxiety-like withdrawal symptoms in C57Bl/6J mice. During acute withdrawal from alcohol and/or nicotine (approximately 24 hours after cessation), a subset of mice underwent the marble burying task in order to measure withdrawal-induced anxiety-like behavior. Mice were housed in individual cages and left for 20 minutes with 20 opaque black marbles spaced evenly in the cage bedding. Marbles buried to at least ⅔ depth were scored as buried by 3 blinded experimenters, independently. A 2-way between subjects ANOVA revealed no significant interaction between or main effects of sex and condition on marbles buried, though there was a trend towards significance for sex ($p=0.17$).