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Effects of Intermittent Alcohol and Nicotine Cc-consumption in C57BL/6J Mice

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Effects of Intermittent Alcohol and Nicotine Co-consumption in C57BL/6J Mice

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Presley Elizabeth Fletcher

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Abstract

Two of the world's leading causes of preventable deaths include the use of alcohol and tobacco. While independently these substances have negative consequences, they are often used in combination. For instance, those who are dependent on nicotine are more likely to engage in hazardous drinking and/or have a dependence on alcohol and vice versa. As different methods of consuming nicotine become more normalized, there is a concern of associated harmful alcohol consumption being that both substances are widely available and rewarding. The goal of the current study was to understand the relationship between nicotine and alcohol and the behavioral effects of co-dependence in an animal drinking model. In the current study, C57BL/6J mice underwent intermittent access to a two-bottle drinking paradigm to investigate physical dependence on alcohol, nicotine, or a combination of both. The concentration of alcohol was increased weekly while the concentration of nicotine remained constant. After the consumption period, mice were then subjected to an open field test (OFT) 24 hours after removal of the test bottles to examine the anxiety-like behavior exhibited during withdrawal from these substances. In all concentrations examined, there was a high preference and increased consumption for alcohol compared to the nicotine-only group. The OFT failed to illustrate significant group differences to demonstrate withdrawal. The current data underscores the complicated nature of alcohol and nicotine co-consumption and withdrawal.

Effects of Intermittent Alcohol and Nicotine Co-consumption in C57BL/6J Mice

Nicotine and alcohol are two of the most commonly abused substances, as they are widely available and they each provide rewarding effects. Tobacco, and resulting nicotine dependence, independently is the leading preventable cause of death worldwide (Locklear, et al. 2012). Alcohol is also responsible for approximately 140,000 deaths per year in the United States alone (Centers for Disease Control and Prevention, 2022. However, smokers are more likely to meet the criteria for Alcohol Use Disorders (AUD), and the use of tobacco contributes to a heightened risk of harmful alcohol consumption or hazardous drinking (Roberts et al., 2018). Interestingly, this applies to e-cigarettes as well, a newer area of study as electronic cigarette use becomes more popular with young adults. Current e-cigarette users are more likely to participate in heavy drinking than peers who do not use e-cigarettes (Roberts, et al., 2018). However, it is important to note that not only are alcohol dependent individuals more likely to be dependent on nicotine as well, but nicotine dependent users are also more likely to be dependent on alcohol (Schlaepfer, et al., 2008). There is a clear comorbidity between these two widely available and harmful substances.

As nicotine and alcohol are often used in combination with one another, there is a potential amplification to the serious health consequences associated, such as the development of cardiovascular diseases, gastric ulcers, and multiple cancers from heavy use (Hurley, et al., 2012). The popular combined use of alcohol and nicotine can possibly be explained by the alleviation of adverse effects of one drug by the other. It appears that certain properties of alcohol (i.e., sedation) may be counteracted by the stimulatory effects of nicotine (Hurley, et al., 2012). The antagonizing effects of nicotine on the properties of alcohol can almost encourage co-users to drink more alcohol, resulting in overall poorer health and treatment outcomes with

chronic usage. It is also suggested that there is an additive effect in the feelings of reward, seeing as up to 90% of individuals who struggle with alcohol addictions also engage in smoking cigarettes (Batel et al., 1995). There is a clear motivation to consume these substances simultaneously despite the serious negative consequences associated with the co-consumption.

Alcohol has a myriad of effects on various neurotransmitters - or chemical messengers - in the brain, including an impact on one of the most important targets for rewarding stimuli, the mesocorticolimbic dopamine (DA) reward system. Overall, this system's role is to regulate mood, emotional responses, and reward-based behaviors. Within this circuit, the ventral tegmental area (VTA) contains dopamine-, glutamate-, and GABA-releasing neurons or nerve cells that project to many cortical and forebrain structures such as the nucleus accumbens, ventral pallidum, amygdala, and the medial prefrontal cortex (Yamaguchi, Wang, Li, Ng, & Morales, 2011). The nucleus accumbens, in particular, is specifically implicated in the emotional and cognitive behavioral changes induced by reward. Within the mesocorticolimbic DA network, the administration of alcohol facilitates the release of DA within these critical brain areas such as the VTA (Hurley, Taylor, & Tizabi, 2012). Increases in DA signaling have proven critical for the acquisition and development of alcohol reinforcement (Doyon et al., 2013). Indeed, alcohol has a variety of effects on the brain, including on the mesocorticolimbic DA reward pathway, that contribute to the widespread use and abuse of the drug.

Nicotine, in contrast, does not have the wide variety of targets that alcohol has; rather, nicotine specifically targets the nicotinic acetylcholine receptors (nAChRs). The nAChRs are ligand-gated ion channels that have high affinity for nicotine which modulate the release of DA and GABA among other neurotransmitters, hence their rewarding properties when activated (Schlaepfer, Hoft, & Ehringer, 2008). The initial exposures of acute nicotine induce a persistent

increase in the synaptic process of glutamatergic impulses towards DA neurons, thereby increasing glutamate release. Through this sequence working throughout the brain, changes underlying learning and memory develop which specialists link to the cultivation of environmental cues for the drug use (Doyon et al., 2013). Importantly, it appears that nAChRs are also stimulated upon administration of alcohol in order to further promote the release of DA within the VTA (Hurley, Taylor, & Tizabi, 2012). While individually, both nicotine and alcohol have been found to increase DA and lead to positive reinforcement, the molecular mechanisms within the reward pathway and overlapping actions at nAChRs likely contribute to the popular combined use of both of these substances.

Taking into consideration the high rate of comorbidity of alcohol and nicotine use among the human population, there is an urgent public health issue that needs to be better understood regarding the effects of this combination of abused substances. In order to go about addressing such concerns, many studies have used animal models to research these substances; however, most of these models investigate the effects of either nicotine or alcohol alone rather than in combination. Animal models commonly utilize rodents when studying nicotine and alcohol abuse because these organisms undergo comparable behavioral and neurophysiological development to humans (Slawecki, Thorsell, & Ehlers, 2004). Such models can provide insight into the neurocircuitry related to dependence and for discovering neurochemical pathways affected by substances (Thiele & Navarro, 2014). Rodent models also provide paradigms that allow for large scale investigations that may otherwise be too costly or laborious, such as genetic screens (Locklear, et al., 2012).

While rodent models offer many advantages when it comes to studying substances of abuse as discussed above, one drawback is often experimenter administered or involuntary

administration of the drug(s). By using more active, voluntary consumption models that give rodents intermittent access to drugs, the pharmacological and behavioral effects assessed are related to more relevant brain regions in terms of decision making processes involved in substance use (O'Rourke, et al., 2016). Intermittent access paradigms attempt to mimic the gradual increase in general alcohol drinking behaviors to excessive alcohol consumption in an easy to follow protocol leading to voluntary consumption of high quantities of alcohol and alcohol dependence/withdrawal (Carnicella, Ron, & Barak, 2014). While this paradigm has been shown to be useful in investigating the neurobiological consequences of large quantities of alcohol, relatively few models exist to study the consequences of varying amounts of nicotine or the combination of alcohol and nicotine.

The main objective of this study is to further examine the relationship between nicotine and alcohol co-consumption. This study will use C57BL/6J male and female mice as this strain is considered to be "high-drinking" and shift intake according to motivation or compulsion (Weera et al., 2018). In a "Drinking in the Dark" procedure, mice tend to elicit high levels of ethanol consumption as well as pharmacologically relevant blood ethanol levels (Thiele & Navarro, 2014). Regarding the co-consumption of alcohol and nicotine, intermittent access paradigms can examine the interactions between nicotine and alcohol relevant to the comorbid addiction found in humans (O'Rourke, et al., 2016). Using an intermittent access voluntary model, male and female C57BL/6J mice were presented with alcohol, nicotine, both, or neither. After approximately 6 weeks of access, each mouse's withdrawal behavior (i.e. anxiety-like behavior) was measured in order to determine the animals' dependence on the substances provided. I hypothesized that the mice will develop a dependence on the substances in which the co-

consumption of alcohol and nicotine induces greater consumption levels and worse withdrawal outcomes compared to the substances alone.

Method

Subjects

The subjects in this research are 24 adult male (n=12) and female (n=12) C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME, USA). Upon the beginning of experimentation, mice were 8 weeks old and housed individually. For the duration of the experiment, all mice lived in a 12h:12h reverse light/dark cycle and had continuous access to food and water. The mice were weighed once per week throughout the experiment. All animal care was carried out in accordance with the National Institutes of Health Office of Laboratory Animal Welfare as well as a protocol approved by the Institutional Animal Care and Use Committee at Butler University.

Drugs & Chemicals

Nicotine hydrogen tartrate salt and 190 proof ethanol were purchased through Sigma (St. Louis, MO, USA). In order to obtain the desired concentrations, tap water was mixed with the alcohol and nicotine.

Experimental Procedures

Each mouse was randomly assigned to one of four groups (control, alcohol alone, nicotine alone, and a combination of alcohol + nicotine) in a two bottle choice intermittent access paradigm. In each of the three experimental groups, singly-housed mice were presented with a bottle of tap water along with a bottle of alcohol, nicotine, or alcohol + nicotine; the control group was presented with two bottles of tap water. Experimental drug bottles were accessible for

24 hours each Monday, Wednesday, and Friday. All bottles were weighed before and after each session, and the position of the drug bottles was switched every two days to control for side preferences.

Behavioral Effects

An open field test was utilized in order to observe anxiety-like behavior in the mice following acute nicotine and/or alcohol withdrawal. The open field test consisted of an enclosed area representing either a square or a circular shape with walls of sufficient height to prevent the mice from escaping. Within this space, different types of motor activity were recorded within a specific time segment of ten minutes. Behaviors measured include ambulation, latency, and rearing (Seibenhener & Wooten, 2015). This test was utilized to examine whether mice are experiencing increased anxiety-like behavior due to the withdrawal effects of alcohol and/or nicotine.

Statistical Analysis

Consumption and Preference: In order to obtain the raw consumption data, the values were first transformed by subtracting the average value of a "leak" control bottle. This control bottle was placed in a cage that was treated exactly like the other cages except there were no mice present. This measure controlled for any potential leakage or evaporation that occurred over time. This value was measured at the same time as the other bottle weights being recorded. Consumption values were transformed based on the percentage of alcohol or nicotine present in the bottle at the time of measurement, density of substance, and the weight of each individual animal. Values were then averaged over the number of days for each concentration producing an average daily alcohol consumption (g/kg/day) and an average daily nicotine consumption (g/kg/day) for each mouse. In order to calculate the percent preference for the alcohol or

nicotine, the number of mls of alcohol or nicotine consumed were divided by the total mls of fluid consumed and then multiplied by 100. Data was analyzed using GraphPad Prism version 8.0 statistical software.

Open Field Test (OFT): The amount of time each mouse spent in the middle 50% of the arena was calculated and averaged within each group. The total distance traveled was also measured for each mouse as well as averaged across conditions. In order to assess the treatment and effects of sex, a 2 X 4 between-subjects ANOVA was conducted. In the case where the effects of sex were absent, the data from both sexes were combined.

Results

Nicotine Only

Consumption and Preference

Mice (n=6, 3 males and 3 females) were presented with a bottle containing 30µg/ml every other 24 hours for four consecutive weeks. As shown in Figure 1, there were no statistically significant nicotinic consumption or preference results for either sex ($p>0.1$). The average preference ratio was around 0.5, indicating no difference in preference between the nicotine bottle and the control bottle (Figure 1B). There was also a lack of significant interactions between sex and time with consumption values varying for individual mice.

Alcohol Only

Consumption

Mice (n=6, 3 males and 3 females) were presented with a bottle of alcohol every other 24 hours. Each Monday over the course of four weeks, the concentration increased (3%, 6%, 10%, 20%). It was found that as the concentration increases, the consumption levels also increase

especially with the female mice. At the highest concentration of alcohol, females drank up to 40mg/kg/day (Figure 2A). A two-way ANOVA revealed a significant main effect of concentration/time ($F(2.183, 8.732) = 70.40$, $p<0.0001$) and a significant interaction of concentration and sex ($F(11, 44) = 5.298$, $p<0.0001$). The main effect of sex was not significant ($F(1, 4) = 7.272$, $p=0.0543$).

Preference

The alcohol preference ratios compared to water for both sexes remained consistently high across each concentration, averaging around 0.9 (Figure 2B). There were no main effects of either concentration or sex, but a two-way ANOVA did reveal a significant concentration and sex interaction ($F(11, 44) = 4.454$, $p<0.001$).

Alcohol + Nicotine

Consumption

Mice (n=6, 3 males and 3 females) were presented with a bottle containing 30µg/ml nicotine and alcohol concentrations every 24 hours, with alcohol concentrations increasing every week over the course of four weeks (3%, 6%, 10%, 20%). Female mice drank significantly more than male mice to the point of separate analysis. Consumption levels increased over time for both sexes, but remained overall consistent (Figure 3A-B). Separate two-way ANOVAs were conducted on each sex in order to simplify results and due to known consumption differences between males and females. Two-way ANOVAs found a significant main effect of substance (alcohol/nicotine) concentration (Males: $F(1, 48) = 278.2$, $p<0.0001$; Females: $F(1, 48) = 228$, $p<0.0001$), a significant main effect of time (Males: $F(11, 48) = 28.98$, $p<0.0001$; Females: $F(11, 48) = 25.79$, $p<0.0001$), and a significant interaction of concentration and time (Males: $F(11, 48) = 25.99$, $p<0.0001$; Females: $F(11, 48) = 24.42$, $p<0.0001$).

Preference

Across all weeks, the preference ratio for the alcohol + nicotine bottle remained consistently high (0.9) for both sexes (Figure 3C). A two-way ANOVA revealed no significant main effect for concentration or sex nor a significant interaction between concentration and sex.

Comparison of Alcohol Consumption Levels

Males

A two-way ANOVA compared alcohol consumption in male mice in the alcohol-consuming groups. The ANOVA revealed no significant main effect of treatment group nor an interaction of treatment group and concentration. There was, however, a significant main effect of concentration ($F(1.344, 5.375) = 64.40$, $p<0.0005$). Male mice demonstrated very similar increases in consumption in the alcohol group and the alcohol + nicotine group across time and concentration, rising up to 28g/kg/day in week 4 (Figure 4A).

Females

A two-way ANOVA compared alcohol consumption in female mice in the alcohol-consuming groups. Similar to what was observed in male mice, the ANOVA revealed no significant main effect of treatment group nor an interaction of treatment group and concentration. There was, however, a significant main effect of concentration ($F(3.390, 13.56) = 68.58$, $p<0.0001$). Female mice demonstrated very similar increases in alcohol consumption for both the alcohol + nicotine and alcohol-only treatment groups across time and concentration with values rising up to 40g/kg/day in Week 4 (Figure 4B).

Comparison of Nicotine Consumption Levels

Males

A separate two-way ANOVA compared nicotine consumption in male mice in nicotine-consuming groups. The ANOVA revealed no significant effect of concentration/time nor a significant interaction. However, there was a significant main effect of treatment group where male mice in the alcohol + nicotine treatment group had significantly greater consumption levels compared to the nicotine-only treatment group with values reaching up to 5.2mg/kg/day in Week 3 (Figure 4C; $F(1, 4) = 17.15$, $p<0.05$).

Females

A two-way ANOVA compared nicotine consumption in female mice in the nicotine-consuming groups. Similar to what was observed in male mice, the ANOVA revealed no significant effect of concentration/time nor a significant interaction. However, there was a significant main effect of treatment group where female mice in the alcohol + nicotine treatment group showed significantly greater consumption levels compared to the nicotine-only group with values reaching up to 7mg/kg/day in Weeks 1, 2, and 3 (Figure 4D; $F(1, 4) = 224.1$, $p<0.001$).

Open Field Test

Mice (n=24) underwent the OFT 24 hours after removing the treatment bottle on the final day of Week 4. Total distance traveled was not different across treatment sex, or interaction ($p>0.05$) (Figure 5A). This result allows one to assume all mice traveled throughout the open fields a similar amount, giving validity to the comparison data between time spent in the outside and inside zones. However, the test failed to show any significant differences for time spent in the inside zone for treatment, sex, or interaction ($p>0.05$) (Figure 5C). The test also failed to show any significant differences for time spent in the outside zone for treatment, sex, or interaction ($p>0.05$) (Figure 5B).

Discussion

This study aimed to investigate the comorbid relationship between alcohol and nicotine using an intermittent access paradigm with a two-bottle choice in C57BL/6J mice. The results demonstrated that the mice showed greater preference for the alcohol-nicotine treatment compared to the nicotine-only treatment. There were similar levels in preference ratios comparatively for the alcohol-only treatment and the alcohol-nicotine combined treatment. Interestingly, as the concentration of alcohol increased each week, the consumption levels correspondingly increased as well, especially for the female mice drinking alcohol-nicotine combined. Overall, C57BL/6J mice exhibit greater consumption and preference for nicotine when it is combined with alcohol. Their tendency to increase consumption as the concentration of alcohol increased in both groups suggests dependency. However, the OFT failed to reveal significant withdrawal behaviors.

Contrary to what is commonly seen in humans, the nicotine-only group of mice demonstrated no significant increase in consumption or preference levels compared to the control water bottle (Figure 1), giving no evidence of physical dependency or inclination. Previous literature has also shown that it can be difficult to obtain significant voluntary consumption of nicotine in mice (Kastan, et al., 2016; O'Rourke, et al., 2016); therefore, these results were not surprising. By contrast, the alcohol-only group of mice exhibited significant increases in consumption over time/concentration and preference levels compared to the control water bottle (Figure 2). This includes a significant interaction between concentration and sex, with female mice showing increased consumption at higher alcohol concentrations compared to male mice. This sex-dependent effect has been shown in our lab previously as well as in prior literature (Evans et al., 2021; Hwa et al., 2011). Overall, results from both nicotine-only and alcohol-only groups were as expected based on previous literature.

The current study, however, was interested in the combination of alcohol and nicotine co-consumption in both male and female mice. Thus, the current results demonstrate that the consumption levels increased across time and alcohol concentration, with consistently high preference ratios for both sexes (Figure 3). Female mice often exhibited greater intake levels as the concentration of alcohol increased each week compared to their male counterparts. These data are also consistent with the alcohol-only group findings with similar increases in intake as concentrations of alcohol increased as well as high preference ratios throughout the duration of the study.

When comparing consumption levels across all alcohol- or nicotine-consuming groups, it is evident that the addition of nicotine to alcohol-containing water had little impact on consumption in either males or females (Figure 4A-B). Again, female mice consumed higher amounts of alcohol compared to male mice, but consumption levels across time increased in a similar manner for both sexes. Within the nicotine-consuming groups, the addition of alcohol resulted in significant increases in consumption compared to nicotine-alone, with similar patterns again observed in both male and female mice (Figure 4C-D). There are persistent, yet stark, differences in the levels of consumption with the combined treatment group consuming significantly more than the nicotine-only group. These data suggest that the risk for increased consumption and physical dependency may rise as concentrations of alcohol increase and as alcohol is combined with the consumption of nicotine.

Despite the observed increases in consumption and preference throughout the experiment, the OFT failed to find significant differences in anxiety-like behavior based on either treatment group or sex (Figure 5). However, this was not entirely surprising given the

voluntary aspect of this consumption model. Despite administering the test 24h after removing the last bottle (a time point at which the mice would be expecting the return of the drug treatment bottles), it is impossible to accurately pinpoint the exact timeline of withdrawal in each animal or to know the last time each animal consumed the drug. Given the use of more than one substance, the timelines of withdrawal phases in mice may present differently. Future studies could also take blood samples during the consumption period to ensure each animal had drank enough to significantly increase blood alcohol concentration (BAC) to the point of physical dependency.

Moving forward, there is still much to be discovered surrounding the neurochemical and behavioral effects of alcohol and nicotine, especially given that there is not much research investigating withdrawal of the combination of these substances or using an intermittent access model. Despite keeping the nicotine levels low and consistent throughout the duration of the current study, the mice still found the substance to be aversive compared to the alcohol-only and combined groups. For the purpose of better modeling human consumption patterns, it is important for pre-clinical research to investigate alternative consumption paradigms, including voluntary and experimenter-administered, and various concentrations that mice would be more prone to develop an affinity for and future dependence on.

Future studies should also consider different behavioral testing paradigms and timelines. The timeline of the withdrawal test could be altered - for example 12h or 48h post-bottle removal instead of 24h. Alternatively, instead of using the OFT, observations of somatic signs of withdrawal could allow for a more direct observation apart from locomotive behaviors. The elevated plus maze is another commonly used paradigm to test anxiety-like behavior that could elicit different results. Further, instead of behavioral testing, direct biological measurements (i.e. blood samples or brain slices) could be used to more precisely measure the amount of drug(s)

consumed or to better capture phases of withdrawal. Another aspect of this procedure that should be addressed in future studies regards the short timeline. The experiment was delayed two weeks due to the interference of university construction within an already limited time constraint given by the sponsoring program. There is potentially a longer consumption period necessary to induce problematic dependence and withdrawal for the substances, and therefore provide clearer OFT results.

The current study sought to further probe the comorbidity of alcohol and nicotine using an intermittent access two-bottle choice paradigm in C57BL/6J mice. The results showed increased consumption and preference for nicotine when it is paired with alcohol, suggesting that individuals who co-abuse both nicotine and alcohol may be at risk for consuming larger amounts compared to individuals who abuse nicotine alone. Although the exact timeframe of withdrawal of the two combined substances needs to be further investigated, it is important to consider the comorbidity of these substances going forward as it is so widespread.

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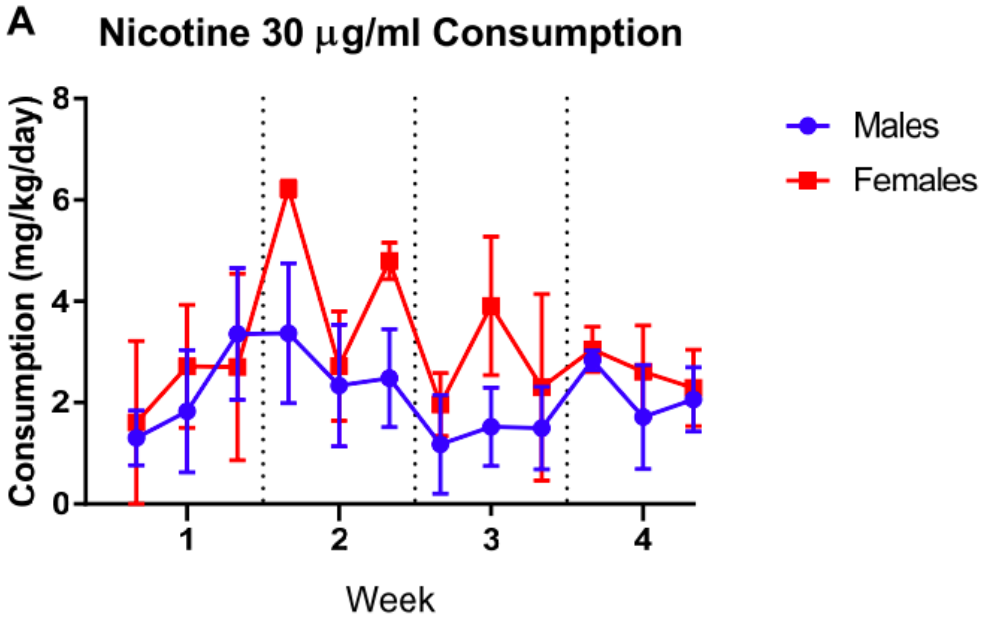
A
Nicotine 30 μg/ml Consumption
Consumption (mg/kg/day)
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2
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6
8
1
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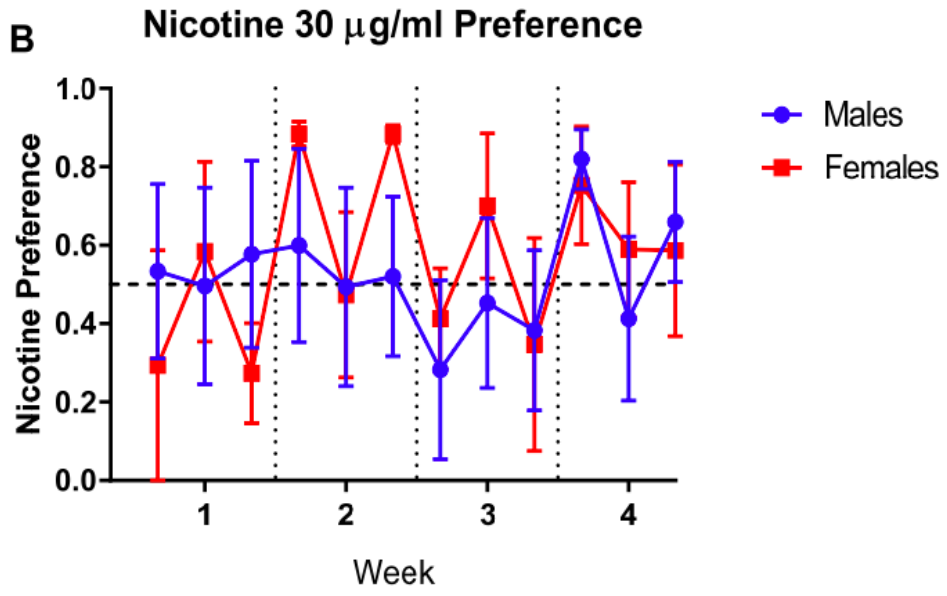
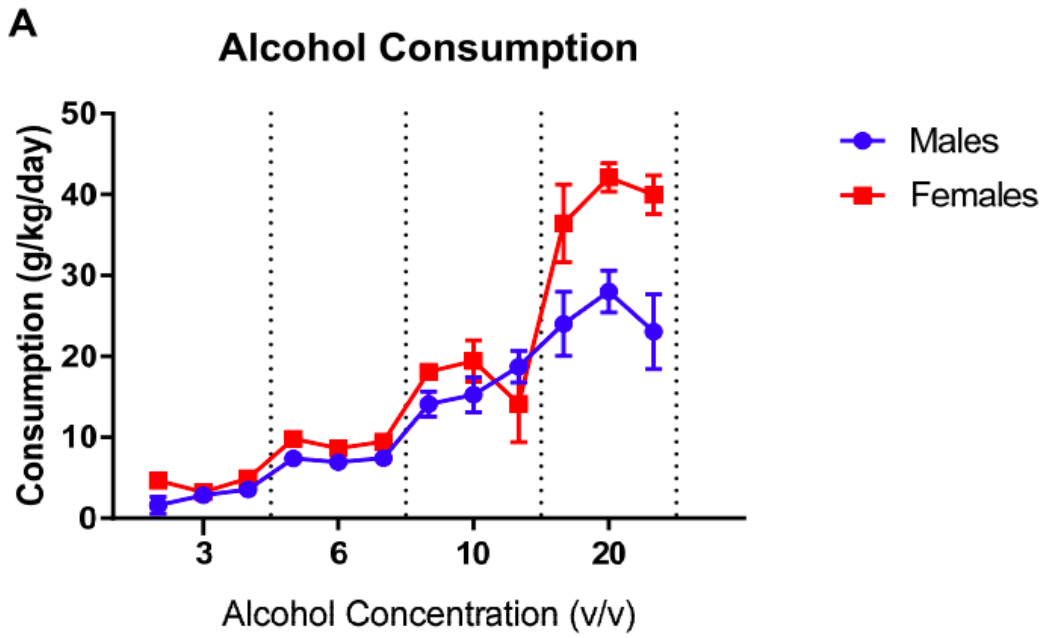


Figure 1. Nicotine consumption in four-week intermittent voluntary access drinking paradigm in male (n=3) and female (n=3) C57BL/6J mice. Two-Way ANOVA found no significant effects. A. Consumption of nicotine (30µg/ml) for males and females across time. B. Preference ratios of nicotine (30µg/ml) for males and females over water.



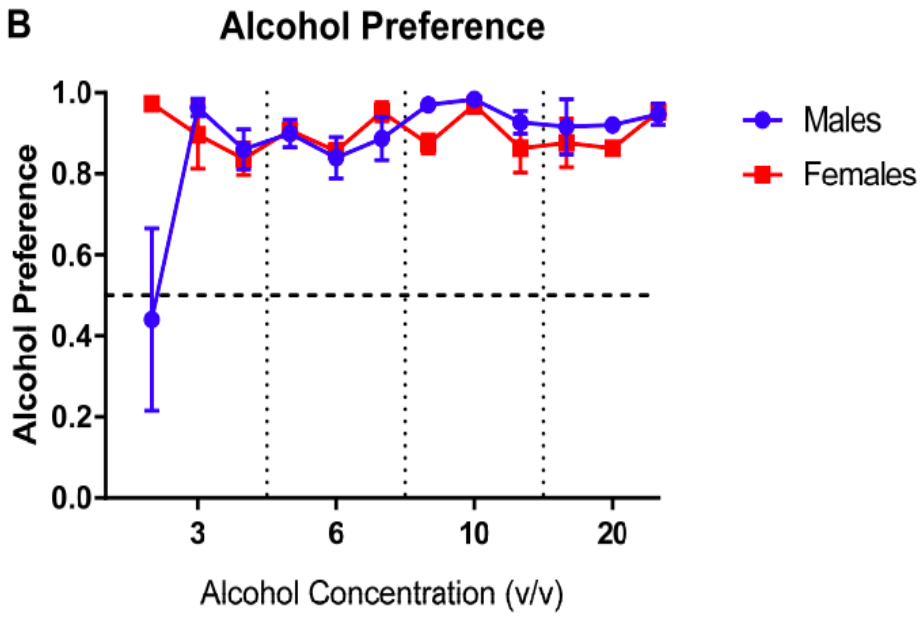


Figure 2. Alcohol consumption and preference in four-week intermittent access voluntary drinking paradigm in male (n=3) and female (n=3) C57BL/6J mice. A. Two-Way ANOVA revealed a significant main effect of concentration/time ($p<0.0001$) and a significant interaction of concentration and sex ($p<0.0001$). B. Two-Way ANOVA revealed a significant concentration and sex interaction ($p<0.001$).

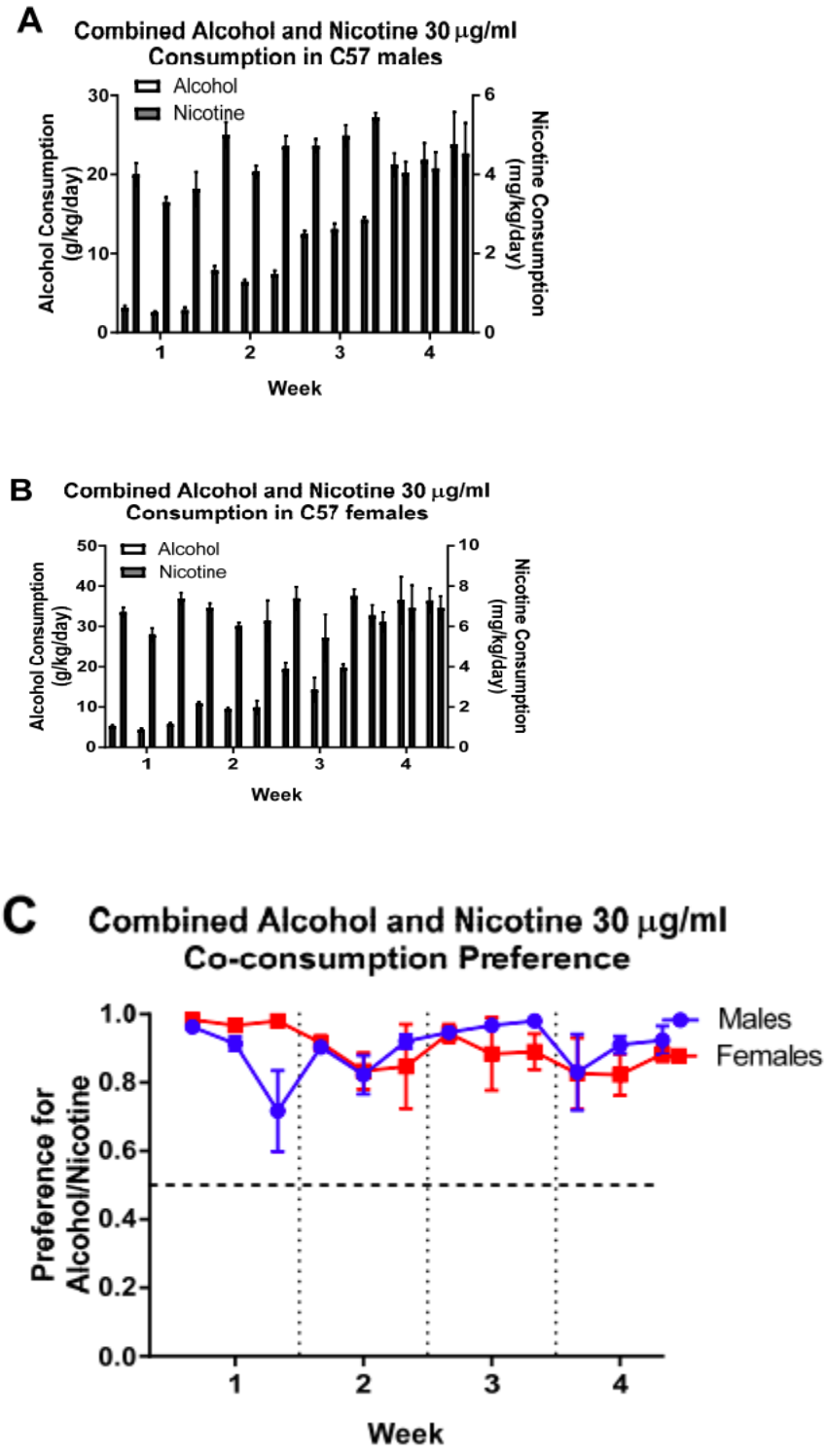


Figure 3. Alcohol and nicotine combined consumption in four-week intermittent voluntary access drinking paradigm in male (n=3) and female (n=3) C57BL/6J mice. A. Two-Way ANOVAs found a significant main effect of substance (alcohol/nicotine) concentration (p<0.0001), a significant main effect of time (p<0.0001), and a significant interaction of

concentration and time (p<0.0001) for female mice. B. Two-Way ANOVAs found a significant main effect of substance (alcohol/nicotine) concentration (p<0.0001), a significant main effect of time (p<0.0001), and a significant interaction of concentration and time (p<0.0001) for male mice. C. Two-Way ANOVA revealed no significant main effect for concentration or sex nor a significant interaction between concentration and sex.

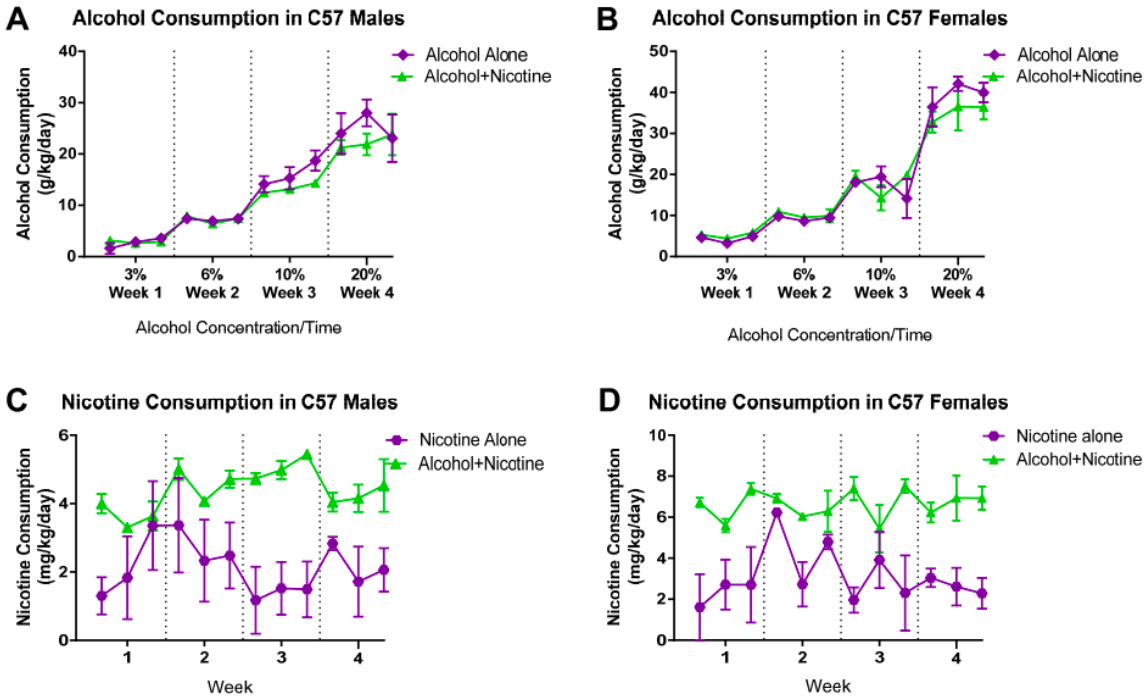
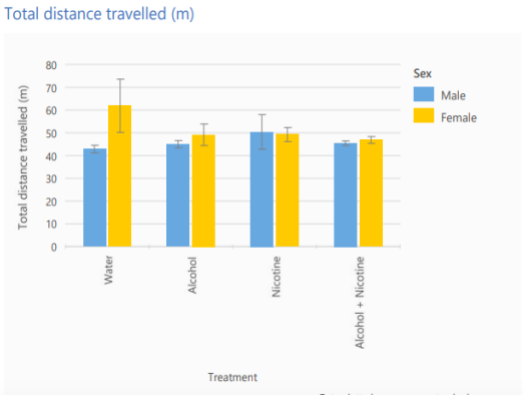
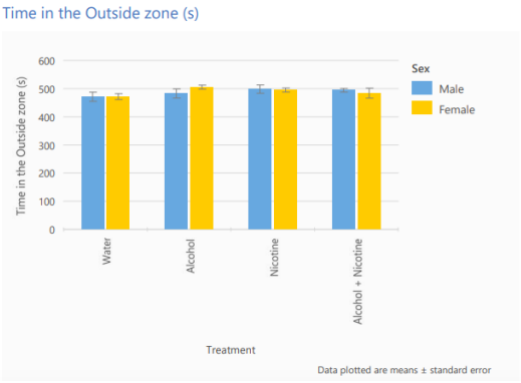


Figure 4. Comparison of consumption levels within alcohol groups (n=12) and nicotine groups (n=12) for C57BL/6J mice. A. Two-Way ANOVA revealed no significant main effect of treatment group nor an interaction of treatment group and concentration. There was a significant main effect of concentration (p<0.0005) for males consuming alcohol. B. Two-Way ANOVA revealed no significant main effect of treatment group nor an interaction of treatment group and concentration. There was a significant main effect of concentration (p<0.0001). C. Two-Way ANOVA revealed no significant effect of concentration/time nor a significant interaction. There was a significant main effect of treatment group where male mice in the alcohol + nicotine treatment group had significantly greater consumption levels compared to the nicotine-only treatment group in Week 3 (p<0.05). D. Two-Way ANOVA revealed no significant effect of concentration/time nor a significant interaction. There was a significant main effect of treatment group where female mice in the alcohol + nicotine treatment group showed significantly greater consumption levels compared to the nicotine-only group in Weeks 1, 2, and 3 (p<0.001).

A.



B.



C.

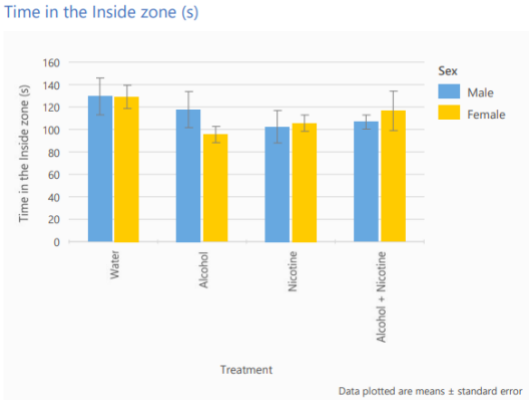


Figure 5. Results from placing C57BL/6J mice (n=24) in Open Field Test after four weeks of intermittent drinking model. A. Total distance traveled throughout the inner and outer zones. B. Time spent in outside zone. C. Time spent in inside zone.