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Voluntary Co-Consumption of Alcohol and Nicotine: Effects of Abstinence, Intermittency, and Withdrawal in Mice

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Abstract

Alcohol and nicotine are often used together, and there is a high rate of co-occurrence between alcohol and nicotine addiction. Most animal models studying alcohol and nicotine interactions have utilized passive drug administration, which may not be relevant to human co-addiction. In addition, the interactions between alcohol and nicotine in female animals have been understudied, as most studies have used male animals. To address these issues, we developed models of alcohol and nicotine co-consumption in male and female mice that utilized voluntary, oral consumption of unsweetened alcohol, nicotine and water. We first examined drug consumption and preference in single-drug, sequential alcohol and nicotine consumption tests in male and female C57BL/6 and DBA/2J mice. We then tested chronic continuous and intermittent access alcohol and nicotine co-consumption procedures. We found that male and female C57BL/6 mice readily co-consumed unsweetened alcohol and nicotine. In our continuous co-consumption procedures, we found that varying the available nicotine concentration during an alcohol abstinence period affected compensatory nicotine consumption during alcohol abstinence, and affected rebound alcohol consumption when alcohol was re-introduced. Consumption of alcohol and nicotine in an intermittent co-consumption procedure produced higher alcohol consumption levels, but not nicotine consumption levels, compared with the continuous co-consumption procedures. Finally, we found that intermittent alcohol and nicotine co-consumption resulted in physical dependence. Our data show that these voluntary co-consumption procedures can be easily performed in mice and can be used to study behavioral interactions between alcohol and nicotine consumption, which may better model human alcohol and nicotine co-addiction.

1. INTRODUCTION

Alcohol and nicotine addiction are highly co-morbid. Alcohol and nicotine are often used together, with studies showing up to 80–90% of individuals with an alcohol addiction are also smokers (Burling and Ziff, 1988; DiFranza and Guerrera, 1990; Batel et al., 1995). Alcohol and nicotine addiction are highly heritable and share common genetic factors (Swan et al., 1996; True et al., 1999) and molecular mechanisms, such as involvement of the nicotinic acetylcholine receptors (Hendrickson et al., 2013), illustrating a biological basis for alcohol and nicotine co-addiction.

Alcohol and nicotine co-use can result in complex interactions, and can produce additive effects at the behavioral (Truitt et al., 2015) and molecular level (Engle et al., 2015). For example, in men and women that use alcohol and nicotine together, alcohol itself or the presentation of alcohol cues increases the urge to smoke (Cooney et al., 2003; King et al., 2009). The interactions between alcohol and nicotine are an integral part of alcohol and nicotine co-addiction; however, most animal models are unable to capture these interactions because very few animal models employ voluntary self-administration of both alcohol and nicotine. The vast majority of animal models either examine alcohol and nicotine consumption in separate groups, which is not clinically relevant to co-addiction, or examine voluntary consumption of one drug with non-contingent administration of the other drug. Non-contingent or passive administration of drugs has been shown to result in different effects compared with voluntary administration, such as altered dopamine (Orejarena et al., 2009) and epinephrine release (Donny et al., 2000), and nicotinic receptor expression (Metaxas et al., 2010). It is possible that models using passive administration may find behavioral and pharmacological effects that are not clinically relevant to human co-addiction. In addition, sex differences in the interactions between alcohol and nicotine consumption are understudied, as most work has been performed in male animals.

Studying alcohol and nicotine co-consumption in animals can be labor intensive and technically challenging if intravenous nicotine administration is utilized. Very few groups have performed studies of voluntary oral alcohol and intravenous nicotine self-administration in male rats (Lê et al., 2010; Lê et al., 2014; Scuppa et al., 2015). Due to the technical challenges of intravenous self-administration in mice, nicotine consumption is most often studied using voluntary oral consumption procedures (Klein et al., 2004; Lee and Messing, 2011; Locklear et al., 2012). Alcohol consumption has long been studied using a variety of oral consumption procedures in mice (Rhodes et al., 2005; Hwa et al., 2011; Lee et al., 2014). Mice also have the advantage of being more amenable to genetic manipulations compared with rats. However, there are no models of voluntary alcohol and nicotine co-consumption in mice.

To address these issues, we have developed several alcohol and nicotine co-consumption procedures in mice. We utilized chronic, voluntary co-consumption of alcohol and nicotine in male and female C57BL/6 and DBA/2J mice, in which the mice are presented with the choice of alcohol, nicotine and water. In this study, we first compared sequential alcohol and nicotine consumption with simultaneous co-consumption of alcohol and nicotine. In our co-consumption model, we examined the effect of alcohol abstinence and reinstatement on the consumption of both drugs. We also compared the amount of drug consumed between an intermittent and a continuous co-consumption procedure. Lastly, to determine whether chronic co-consumption of alcohol and nicotine resulted in physical dependence, we examined the development of somatic withdrawal signs in mice. Our work here shows that voluntary alcohol and nicotine co-consumption procedures can be readily performed in mice, and can be used to induce and investigate addiction-related behaviors.

2. MATERIALS AND METHODS

2.1. Animals and Drugs

Adult male and female C57BL/6 and DBA/2J mice were a minimum of 56 days old in our experiments. Mice were purchased from Jackson Laboratory (Sacramento, CA) and acclimated to our facility for a minimum of six days before behavioral experiments. All mice were group housed in standard cages under a 12-hour light/dark cycle until the start of behavioral experiments, when they were individually housed. For all experiments, food and water were freely available at all times. All animal procedures were in accordance with the Institutional Animal Care and Use Committee at the University of Minnesota, and conformed to NIH guidelines (National Research Council Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2010).

Alcohol (Decon Labs, King of Prussia, PA), and nicotine tartrate salt (Acros Organics, Thermo Fisher Scientific, Waltham, MA) were mixed with tap water to the concentrations reported for each experiment. The concentrations of nicotine reported are free base, and the nicotine solutions were not filtered or pH adjusted. The alcohol and nicotine solutions were not masked with any sweetener.

2.2. Experiment 1: Sequential Alcohol and Nicotine Two-Bottle Choice Consumption Tests

The objective of this experiment was to assess the levels of alcohol and nicotine consumption in C57BL/6 and DBA/2J mice when alcohol and nicotine were presented sequentially. Ten drug-naïve male and female C57BL/6 and DBA/2J mice were singly housed in double grommet cages. For the alcohol consumption portion, mice underwent a continuous 2-bottle choice alcohol consumption procedure that we have previously used (Lee et al., 2014), and were presented with a bottle of tap water and a bottle of tap water containing increasing concentrations of alcohol: 3, 6, 10, 14 and 20% v/v. Each concentration was presented for 4 days. The bottles were weighed every 2 days, and the positions of the bottles were alternated to account for side preferences. At the conclusion of the alcohol consumption portion, mice were maintained in double grommet cages and presented with only water for 2 weeks before beginning the nicotine consumption portion. Mice were presented with a bottle of tap water and a bottle of tap water containing increasing concentrations of nicotine: 30, 40 and 50 µg/mL w/v. Each concentration was presented for one week, as we have found that nicotine consumption levels stabilize within one week. The bottles were weighed every second day and the positions of the bottles were alternated to account for side preferences. All solutions were refreshed every 3–4 days. The mice were weighed once a week throughout the entire study.

2.3. Experiment 2A and 2B: Co-consumption of Alcohol and Nicotine in a Continuous Access Three-Bottle Choice Test

The objective of these experiments was to assess the levels of alcohol and nicotine consumption when they were presented simultaneously, and to test the hypotheses that nicotine consumption would increase during a period of alcohol abstinence, and that re-introduction of alcohol after abstinence would result in higher levels of consumption compared with consumption prior to abstinence. For each experiment, fifteen drug-naïve

male and female C57BL/6 mice were singly housed in custom cages that accommodated three drinking bottles (Ancare, Bellmore, NY). Mice were presented with three bottles containing alcohol, nicotine or water, with each set of concentrations presented for one week. Drug concentrations slowly escalated over the first three weeks before initiating the alcohol abstinence phase. The maximum nicotine concentration was 30 µg/mL to ensure adequate nicotine preference in male mice and to obtain similar nicotine consumption levels between sexes. The maximum alcohol concentration was 20% to obtain similar alcohol consumption levels between sexes. The concentrations were: for week 1 – 3% alcohol and 5 µg/mL nicotine, for week 2 – 10% alcohol and 15 µg/mL nicotine, and for week 3 – 20% alcohol and 30 µg/mL nicotine. The design for Experiment 2A and 2B diverged in week 4. In Experiment 2A, alcohol was removed and 30 µg/mL nicotine was presented along with the water bottle. In week 5, the 20% alcohol bottle was re-introduced along with 30 µg/mL nicotine and water. In Experiment 2B, the concentration of nicotine was dropped from 30 to 20 µg/mL during the alcohol abstinence week. This decrease in nicotine concentration was done to ensure that we did not encounter a ceiling effect for nicotine consumption and preference for the male C57BL/6 mice, since our single-drug nicotine consumption test showed that 30 µg/mL elicited the highest preference among all concentrations tested for the male C57BL/6 mice. In addition, we wanted to determine the effect of lowering the concentration of nicotine on subsequent consumption. In week 5, the 20% alcohol bottle was re-introduced, along with 20 µg/mL nicotine and water. All solutions were refreshed every 3–4 days, the weights of the bottles were measured every second day, and the mice were weighed once a week.

2.4. Experiment 3: Co-consumption of Alcohol and Nicotine in an Intermittent Access Three-Bottle Choice Test

The objective of this experiment was to assess the levels of alcohol and nicotine consumption when the drugs were presented simultaneously in an intermittent schedule, with the hypothesis that intermittency would increase the amount of drug consumed. Fifteen drug-naïve male and female C57BL/6 mice were singly housed in custom cages that accommodated three drinking bottles. The intermittent access co-consumption procedure was based on the alcohol intermittent access procedure by Hwa and colleagues (Hwa et al., 2011). Alcohol, nicotine and water bottles were presented for 24-hours each day on Monday, Wednesday and Friday. During week 1, which is an escalation week that starts with low drug concentrations, the concentration of alcohol and nicotine increased for every session, beginning with 3% alcohol and 5 µg/mL nicotine on Monday, 6% alcohol and 10 µg/mL nicotine on Wednesday, and 10% alcohol and 15 µg/mL nicotine on Friday. For the subsequent weeks 2–4, mice received 20% alcohol and 30 µg/mL nicotine every Monday, Wednesday and Friday. At all other times, the mice only had access to water. The bottles were weighed before and after every session, and the positions of the bottles were alternated for every session. The solutions were refreshed every 3–4 days. The mice were weighed once a week for the duration of the study.

2.5. Experiment 3: Measurement of Somatic Withdrawal Signs in the Intermittent Access Co-consumption Test

For baseline behavior measurements, fifteen male and female C57BL/6 mice were individually placed in clean, standard cages with a micro-isolator top and videotaped for 15 minutes, prior to beginning the intermittent co-consumption procedure outlined in Section 2.4. At the end of the 4-week intermittent co-consumption test, 24 hours after access to the alcohol and nicotine bottles had ended, the mice were individually placed in clean, standard cages and videotaped for 15 minutes. Somatic withdrawal signs were scored by a reviewer blinded to when the videotape was recorded. The withdrawal signs were chosen based on studies that have examined alcohol and nicotine withdrawal in mice (Damaj et al., 2003; Jackson et al., 2008; Locklear et al., 2012; Perez and De Biasi, 2015) and included grooming, front paw tremors, wet dog shakes, head shakes, tail rattling, chattering, hunched position, hind foot scratching/shaking, body twitching, jumping forwards, escape attempts (jumping while rearing), cage scratching, digging, licking/sniffing cage, backing up and mastication. The incidence of each somatic withdrawal sign that occurred during the 15 minutes was noted. The incidences of somatic withdrawal signs that were seen in less than half the mice (front paw tremors, wet dog shakes, head shakes, tail rattling, chattering and hunched position) were compiled to form a minor withdrawal sign score. All other withdrawal signs were scored separately.

2.6. Statistical Analysis

For each experiment, the average daily alcohol (g/kg/day) and nicotine consumption (mg/kg/day) for each concentration was calculated based on the weights of the bottles, the density of the solution (for the alcohol bottles only), and the weight of the individual mouse. The percent preference for the alcohol, nicotine or water bottle was calculated as the weight of the bottle divided by the weights of all three bottles combined $\times 100$. Outliers were detected using the ROUT test. Consumption and preference were analyzed using two-way repeated measures ANOVA with a Sidak's multiple comparison post-hoc test, or using a Student's t-test. Withdrawal scores were calculated by subtracting the baseline score from the score recorded after chronic consumption. Withdrawal scores were compared with a hypothetical score of zero, representing no withdrawal, using a one-sample, two-way Student's t-test. All analyses were calculated using Prism 6.0 (GraphPad, La Jolla, CA).

3. RESULTS

3.1. Experiment 1: Sequential Alcohol and Nicotine Two-Bottle Choice Consumption Tests

Drug-naïve male and female C57BL/6 and DBA/2J mice underwent an alcohol 2-bottle choice test, followed by two weeks of water consumption only, followed by a nicotine 2-bottle choice test. C57BL/6 mice consumed more alcohol and had a higher preference for alcohol compared with DBA/2J mice. Male C57BL/6 mice had higher levels of average daily alcohol consumption at the 10, 14, and 20% alcohol concentrations (Fig. 1A) [$F_{\text{strain} \times \text{concentration}} (4,72)=13.20, P<0.0001$; post-hoc tests $P<0.0001$ for 10, 14 and 20% concentrations], and had a greater alcohol preference at all concentrations compared with male DBA/2J mice (Fig. 1B) [$F_{\text{strain} \times \text{concentration}} (4, 72)=7.760, P<0.0001$; post-hoc tests: 3% $P<0.0001$, 6% $P<0.01$, 10% $P<0.0001$, 14% $P<0.0001$ and 20% $P<0.01$]. Female

C57BL/6 mice had higher levels of average daily alcohol consumption at 6, 10, 14, and 20% alcohol concentrations (Fig. 1A) [$F_{\text{strain} \times \text{concentration}} (4, 72)=9.283, P<0.0001$; post-hoc tests $P<0.0001$ for 6, 10, 14 and 20% concentrations], and greater alcohol preference at all concentrations compared with female DBA/2J mice (Fig. 1B) [$F_{\text{strain} \times \text{concentration}} (4, 72)=6.061, P<0.0001$; post-hoc tests: 3, 6, 10 and 14% all $P<0.0001$, 20% $P<0.01$].

Within the C57BL/6 strain, females had higher levels of average daily alcohol consumption compared with males at the 10 and 14% alcohol concentrations (Fig. 1A) [$F_{\text{sex} \times \text{concentration}} (4, 72)=4.918, P<0.001$; post-hoc tests: 10 and 14% $P<0.01$], but did not have differences in alcohol preference (Fig. 1B) [$F_{\text{sex} \times \text{concentration}} (4, 72)=3.838, P<0.003$; post-hoc tests were $P>0.05$ comparing male and female alcohol preference at 3, 6, 10, 14 and 20% concentrations]. There was also no difference in total fluid consumption, as measured by alcohol and water combined, between the male and female C57BL/6 mice at any alcohol concentration [$F_{\text{sex} \times \text{concentration}} (4, 72)=4.120, P<0.002$; post-hoc tests were $P>0.05$ comparing male and female fluid consumption at 3, 6, 10, 14 and 20% concentrations]. Within the DBA/2J strain, males had higher levels of average daily alcohol consumption at the 6 and 20% alcohol concentrations (Fig. 1A) [$F_{\text{sex} \times \text{concentration}} (4, 72)=14.38, P=0.002$; post-hoc tests $P<0.05$ for 6 and 20% concentrations], and greater alcohol preference at the 6% concentration compared with female DBA/2J mice (Fig. 1B) [$F_{\text{sex} \times \text{concentration}} (4, 72)=21.36, P<0.0001$, post-hoc tests $P<0.0001$ for 6% concentration, non-significant for all other concentrations].

In the nicotine 2-bottle choice test, there was an overall effect of strain for nicotine consumption (Fig. 1C), and for nicotine preference (Fig. 1D), between male C57BL/6 and DBA/2J mice, with male C57BL/6 mice consuming more nicotine and having a greater nicotine preference compared with male DBA/2J mice [nicotine consumption: $F_{\text{concentration} \times \text{strain}} (2, 36)=0.147, P=0.14$; $F_{\text{concentration}} (2, 36)=0.483, P=0.81$; $F_{\text{strain}} (1, 36)=15.85, P=0.01$; nicotine preference: $F_{\text{concentration} \times \text{strain}} (2, 36)=5.494, P=0.10$; $F_{\text{concentration}} (2, 36)=2.999, P=0.27$; $F_{\text{strain}} (1, 36)=17.92, P=0.007$]. Female C57BL/6 mice consumed more average daily nicotine at the 40 and 50 $\mu\text{g/mL}$ concentrations (Fig. 1C) [$F_{\text{strain} \times \text{concentration}} (2, 36)=13.91, P=0.0001$; post-hoc tests $P<0.0001$ for 40 and 50 $\mu\text{g/mL}$], and had greater nicotine preference at the 40 and 50 $\mu\text{g/mL}$ concentrations compared with female DBA/2J mice (Fig. 1D) [$F_{\text{strain} \times \text{concentration}} (2, 36)=9.186, P=0.004$; post-hoc tests $P<0.0001$ for 40 and 50 $\mu\text{g/mL}$].

Within strain, female C57BL/6 mice had higher levels of average daily nicotine consumption at the 40 and 50 $\mu\text{g/mL}$ nicotine concentrations (Fig. 1C) [$F_{\text{sex} \times \text{concentration}} (2, 36)=14.78, P<0.0001$; post-hoc tests: 40 $\mu\text{g/mL}$ $P<0.05$, 50 $\mu\text{g/mL}$ $P<0.0001$], and greater preference for the 50 $\mu\text{g/mL}$ nicotine concentration compared with male C57BL/6 mice (Fig. 1D) [$F_{\text{sex} \times \text{concentration}} (2, 36)=14.44, P=0.001$; post-hoc tests $P<0.05$ for 50 $\mu\text{g/mL}$]. Female C57BL/6 mice also consumed more total fluid for the 50 $\mu\text{g/mL}$ concentration, but not for the 30 or 40 $\mu\text{g/mL}$ concentrations, compared with male mice [$F_{\text{sex} \times \text{concentration}} (2, 36)=6.812, P=0.01$; post-hoc tests $P<0.001$ for 50 $\mu\text{g/mL}$]. There were no sex differences in nicotine consumption or nicotine preference between male and female DBA/2J mice.

3.2. Experiment 2A and 2B: Co-consumption of Alcohol and Nicotine in a Continuous Access Three-Bottle Choice Test

In both experiments, drug-naïve male and female C57BL/6 mice underwent an escalating 3-bottle choice, continuous access procedure for the first three weeks. During the fourth week, the alcohol bottle was removed but the nicotine bottle remained available, and during the fifth week, the alcohol bottle was re-introduced. Over the entire five weeks for Experiment 2A, female C57BL/6 mice had higher levels of average daily alcohol consumption compared with male mice for each concentration except for 3% [$F_{\text{sex} \times \text{concentration}} (3, 84)=4.747$, $P=0.002$; post-hoc tests: Week 2 10% $P<0.01$, Week 3 20% $P<0.0001$, Week 5 20% $P<0.001$]. There were no sex differences in nicotine consumption between males and females [$F_{\text{sex} \times \text{concentration}} (4, 112)=0.778$, $P=0.41$; $F_{\text{sex}} (1, 112)=0.531$, $P=0.24$; $F_{\text{concentration}} (4, 112)=67.03$, $P<0.0001$] (Fig. 2A and C). There was a sex difference for ethanol preference [$F_{\text{sex} \times \text{concentration}} (3, 84)=3.032$, $P=0.13$; $F_{\text{sex}} (1, 84)=8.154$, $P=0.006$; $F_{\text{concentration}} (3, 84)=20.12$, $P<0.0001$] and nicotine preference [$F_{\text{sex} \times \text{concentration}} (4, 114)=0.507$, $P=0.84$; $F_{\text{sex}} (1, 114)=35.98$, $P<0.0001$; $F_{\text{concentration}} (4, 112)=6.470$, $P=0.003$] for the entire experiment, with females showing greater ethanol preference compared with males, and males showing greater nicotine preference compared with females (Fig. 2B and D). During week 4, we removed the alcohol bottle but maintained access to the 30 $\mu\text{g/mL}$ nicotine and water bottles. For both males and females, nicotine consumption significantly increased during alcohol abstinence, as compared with the previous week when both drugs were available [$F_{\text{sex} \times \text{session}} (1, 28)=0.002$, $P=0.96$; $F_{\text{sex}} (1, 28)=1.480$, $P=0.38$; $F_{\text{session}} (1, 28)=25.06$, $P<0.0001$] (Fig. 2A and C). There was also an increase in nicotine preference during the alcohol abstinence week, and males showed greater nicotine preference compared with females [$F_{\text{sex} \times \text{session}} (1, 28)=0.268$, $P=0.55$; $F_{\text{sex}} (1, 28)=6.451$, $P=0.04$; $F_{\text{session}} (1, 28)=33.85$, $P<0.0001$] (Fig. 2B and D). Water preference also increased during the alcohol abstinence week [$F_{\text{sex} \times \text{session}} (1, 28)=3.933$, $P=0.03$; post-hoc tests: females week 3 to 4 $P<0.0001$; males week 3 to 4 $P<0.01$]. When 20% alcohol was re-introduced in week 5, there was no change in the levels of alcohol consumption and preference, or in nicotine consumption and preference compared with the week prior to alcohol abstinence, suggesting that alcohol abstinence did not result in a rebound consumption of alcohol for either sex [alcohol consumption: $F_{\text{sex} \times \text{session}} (1, 28)=0.205$, $P=0.61$; $F_{\text{sex}} (1, 28)=30.68$, $P=0.0002$; $F_{\text{session}} (1, 28)=1.878$, $P=0.13$; alcohol preference: $F_{\text{sex} \times \text{session}} (1, 28)=0.009$, $P=0.91$; $F_{\text{sex}} (1, 28)=14.90$, $P=0.01$; $F_{\text{session}} (1, 28)=2.374$, $P=0.09$; nicotine consumption: $F_{\text{sex} \times \text{session}} (1, 28)=0.593$, $P=0.46$; $F_{\text{sex}} (1, 28)=7.970$, $P=0.06$; $F_{\text{session}} (1, 28)=1.992$, $P=0.18$; nicotine preference: $F_{\text{sex} \times \text{session}} (1, 28)=0.314$, $P=0.55$; $F_{\text{sex}} (1, 28)=5.578$, $P=0.14$; $F_{\text{session}} (1, 28)=2.340$, $P=0.11$].

To test the effect of reducing the nicotine concentration during alcohol abstinence week, we lowered the concentration from 30 to 20 $\mu\text{g/mL}$ during week 4 in Experiment 2B. Of the three nicotine concentrations presented in the first three weeks, 30 $\mu\text{g/mL}$ elicited the highest levels of nicotine consumption in both males and females, similar to Experiment 2A. Over the entire five-week experiment, female C57BL/6 mice had higher levels of average daily alcohol consumption compared with male mice for each concentration except for 3% [$F_{\text{sex} \times \text{concentration}} (3, 84)=6.705$, $P=0.0001$; post-hoc tests: Week 2 10% $P<0.001$, Week 3 20% $P<0.0001$, Week 5 20% $P<0.0001$] (Fig. 3A and C). Female mice had a higher alcohol

preference compared with male mice [$F_{\text{sex} \times \text{session}} (3, 84)=2.614, P=0.09$; $F_{\text{session}} (3, 84)=40.15, P<0.0001$; $F_{\text{sex}} (1, 84)=10.11, P=0.0001$] (Fig. 3B and D). Female C57BL/6 mice also had higher levels of nicotine consumption [$F_{\text{sex} \times \text{session}} (4, 112)=1.157, P=0.47$; $F_{\text{session}} (4, 112)=46.10, P<0.0001$; $F_{\text{sex}} (1, 112)=2.303, P=0.04$] but no difference in nicotine preference [$F_{\text{sex} \times \text{session}} (4, 112)=2.111, P=0.31$; $F_{\text{session}} (4, 112)=24.60, P<0.0001$; $F_{\text{sex}} (1, 112)=0.589, P=0.41$] compared with male mice (Fig. 3B and D). During the alcohol abstinence week, we found that nicotine consumption decreased in both the male and female mice compared to the previous week when both drugs were available [$F_{\text{sex} \times \text{session}} (1, 28)=0.528, P=0.51$; $F_{\text{session}} (1, 28)=6.254, P=0.03$; $F_{\text{sex}} (1, 28)=5.950, P=0.09$] (Fig. 3A and C), which is in contrast to Experiment 2A. There was no difference in nicotine preference between weeks 3 and 4 when the alcohol bottle was removed [$F_{\text{sex} \times \text{session}} (1, 28)=2.489, P=0.14$; $F_{\text{session}} (1, 28)=0.592, P=0.47$; $F_{\text{sex}} (1, 28)=0.215, P=0.77$], and there was no sex difference in nicotine consumption or nicotine preference (Fig. 3B and D). The preference for water significantly increased between weeks 3 and 4 in both male and female mice [$F_{\text{sex} \times \text{session}} (1, 28)=0.020, P=0.85$; $F_{\text{session}} (1, 28)=49.03, P<0.0001$; $F_{\text{sex}} (1, 28)=0.486, P=0.53$]. When the 20% alcohol bottle was re-introduced in week 5, both male and female mice had increased alcohol consumption compared with the week prior to abstinence [$F_{\text{sex} \times \text{session}} (1, 28)=1.715, P=0.15$; $F_{\text{session}} (1, 28)=10.20, P=0.001$; $F_{\text{sex}} (1, 28)=34.49, P<0.0001$] (Fig. 3A and C), and there was a trend towards an increase in alcohol preference before and after alcohol abstinence [$F_{\text{sex} \times \text{session}} (1, 28)=0.925, P=0.38$; $F_{\text{session}} (1, 28)=4.165, P=0.067$; $F_{\text{sex}} (1, 28)=22.81, P=0.0004$].

3.3. Comparison of Sequential Two-bottle Choice Alcohol and Nicotine Consumption with Three-bottle Choice Continuous Access Alcohol and Nicotine Consumption

We compared the average daily consumption levels of alcohol and nicotine from the 2-bottle choice test in Experiment 1 with the average daily consumption levels for the same alcohol and nicotine concentrations in the 3-bottle choice tests in Experiments 2A and 2B combined, prior to the alcohol abstinence phase, to determine if co-consumption of alcohol and nicotine differed from sequential consumption. In male C57BL/6 mice, presentation of alcohol and nicotine together resulted in lower average daily alcohol consumption for the 10 and 20% concentrations compared with the average daily consumption at those concentrations in the sequential alcohol 2-bottle choice test (Fig. 4A) [$F_{\text{concentration} \times \text{procedure}} (2, 111)=6.241, P<0.0001$; post-hoc tests 10% and 20% $P<0.0001$]. Females did not show any difference in alcohol consumption between the 2- and 3-bottle choice tests (Fig. 4B) [$F_{\text{concentration} \times \text{procedure}} (2, 113)=3.517, P=0.005$; post-hoc tests comparing 2BC to 3BC for 3, 10 and 20% were non-significant, but 3% ethanol was significantly different from 10 and 20% ethanol within the 2BC and 3BC tests]. There was no difference in the average daily nicotine consumption of the 30 $\mu\text{g/mL}$ concentration between the 2- and 3-bottle choice tests in either males ($t=1.310, P=0.20$) or females ($t=0.708, P=0.48$) (Fig. 4C and D).

3.4. Experiment 3: Co-consumption of Alcohol and Nicotine in an Intermittent Three-Bottle Choice Test

Drug-naïve male and female C57BL/6 mice underwent a 3-bottle intermittent access test. Similar to the continuous access 3-bottle choice test described in Experiment 2, the 30 $\mu\text{g/mL}$ nicotine concentration elicited the highest levels of nicotine consumption in male and

female mice. Female mice showed higher average daily alcohol consumption for the 20% alcohol concentration during weeks 2–4 compared with male mice (Fig. 5A and C) [$F_{\text{sex} \times \text{session}} (5, 140)=6.289, P<0.0001$; post-hoc tests: week 2 $P<0.01$, week 3 and 4 $P<0.0001$], but there was no sex difference in alcohol preference (Fig. 5B and D) [$F_{\text{sex} \times \text{session}} (5, 140)=1.532, P<0.52$; $F_{\text{session}} (5, 140)=18.84, P<0.0001$; $F_{\text{sex}} (1, 140)=0.606, P=0.44$]. Female mice also showed higher average daily nicotine consumption during week 3 compared with male mice [$F_{\text{sex} \times \text{session}} (5, 140)=5.225, P=0.03$; post-hoc tests: week 3 $P<0.05$, non-significant for weeks 2 and 4], but there was no sex difference in nicotine preference (Fig. 5B and D) [$F_{\text{sex} \times \text{session}} (5, 140)=5.776, P<0.04$; post-hoc tests: non-significant for weeks 2–4 comparing males and females].

To determine whether chronic, intermittent consumption of alcohol and nicotine resulted in physical dependence, we assessed signs of drug withdrawal before and after the intermittent co-consumption test. At the end of 4 weeks of co-consumption, we measured withdrawal signs 24 hours after removal of the alcohol and nicotine bottles. Male mice showed a significantly higher frequency of minor withdrawal signs (Fig. 6A) ($t=2.550, P=0.02$), and grooming (Fig. 6B) ($t=3.353, P=0.005$). Male mice also showed a trend towards increased body twitches ($P=0.08$, Fig. 6C) and mastication ($P=0.08$, Fig. 6D). Female mice showed different somatic withdrawal signs compared with males, with a significantly higher frequency of hind foot scratching/shaking (Fig. 6E) ($t=2.466, P=0.03$), and a trend towards increased backing up (Fig. 6F) ($P=0.08$). We observed no changes in digging, jumping forwards, cage scratching, licking/sniffing the cage or escape attempts (Fig. 6G–K).

3.5. Comparison of the Continuous Access Three-Bottle Choice Test with the Intermittent Access Three-Bottle Choice Test

We compared the levels of average daily alcohol and nicotine consumption for the 20% alcohol and 30 $\mu\text{g/mL}$ nicotine solutions during the third week of the continuous access 3-bottle choice tests (Experiments 2A and 2B) with the levels of average daily alcohol and nicotine consumption for the same concentrations during weeks 2–4 of the intermittent access 3-bottle choice test (Experiment 3) to determine if intermittency affected drug consumption at equivalent drug concentrations. We found that males had significantly higher average daily alcohol consumption levels ($t=3.006, P=0.007$) (Fig. 7A), and females showed a strong trend towards higher alcohol consumption in the intermittent access test compared with the continuous access test ($t=1.941, P=0.06$) (Fig. 7B). Male mice also showed a strong trend towards decreased nicotine consumption levels in the intermittent access 3-bottle choice test compared with the continuous access test ($t=2.011, P=0.05$) (Fig. 7C). Females did not show significant differences in nicotine consumption levels between the two tests ($t=1.335, P=0.19$) (Fig. 7D).

4. DISCUSSION

In this study, we assessed voluntary, oral alcohol and nicotine co-consumption models in male and female mice using 3-bottle choice procedures. One advantage of our models is that they do not require training, and large numbers of mice can be tested easily. Voluntary co-consumption in 3-bottle choice procedures can capture the behavioral and pharmacological

interactions between alcohol and nicotine that are absent in single consumption tests, forced consumption tests, or tests that involve investigator-administered drugs. The majority of alcohol and nicotine interaction studies have used investigator-administered drugs, such as injection of nicotine or alcohol while allowing self-administration of the other drug. In studies of voluntary alcohol consumption and investigator-administered nicotine in rats and mice, nicotine injections can decrease (Blomqvist et al., 1996; Hendrickson et al., 2009; Hauser et al., 2012), or increase (Lê et al., 2000; Lê et al., 2003; Hauser et al., 2012; Sajja and Rahman, 2012) alcohol consumption depending on the genetic background, as well as the dose, timing and duration of the nicotine injection. In the few studies that have investigated coadministration of intravenous nicotine and oral alcohol in male rats, consumption of both drugs together result in decreased nicotine intake compared with rats self-administering nicotine alone, but self-administration of alcohol and nicotine together did not have an effect on the level of alcohol consumption (Lê et al., 2010; Scuppa et al., 2015). We found that in our continuous access tests of alcohol and nicotine co-consumption, male mice consumed less alcohol when nicotine was available, but the availability of alcohol did not affect nicotine consumption. The differences between our findings and those previously published could be due to several factors, such as the nature of the consumption procedure (operant versus non-operant), the length of exposure to both drugs, the amount of drug consumed, or the species used. The varied effects of alcohol and nicotine combined could also be due to time-dependent changes in alcohol and nicotine interactions, since injections of nicotine initially decrease alcohol consumption in rats, but chronic nicotine injections enhance nicotine consumption (Lê et al., 2000). Nicotine acts on nicotinic acetylcholine receptors that are critical for nicotine reinforcement, particularly those containing α_4 , α_6 , and β_2 subunits (Picciotto et al., 1998; Tapper et al., 2004; Pons et al., 2008). Alcohol can alter ligand-induced nAChR currents, depending on the subunit composition of the receptor (Nagata et al., 1996; Yu et al., 1996; Covernton and Connolly, 1997). Alcohol potentiates the activity of α_2 - and α_4 -containing nAChRs, which is likely due to stabilization of the open channel state of the receptor (Wu et al., 1994; Forman and Zhou, 1999). If nicotine-activated nAChR currents result in nicotine reward and satiety, enhancement of this signal by alcohol could increase nicotine consumption. On the other hand, enhancement of nicotine-activated nAChR currents by alcohol could reduce nicotine consumption if increased satiety reduces the desire for subsequent drug intake.

Although combined alcohol and nicotine use is extremely common, treatment programs do not typically address smoking cessation during alcohol dependence treatment, and individuals dependent on both substances may not prefer simultaneous treatment (Kodl et al., 2006; Fucito and Hanrahan, 2015). However, simultaneous treatment can reduce alcohol consumption and increase alcohol abstinence (Gulliver et al., 2006; Cooney et al., 2015). One of our goals was to investigate nicotine consumption during alcohol abstinence and reinstatement, and the effects of alcohol abstinence on subsequent alcohol and nicotine co-consumption, which could help inform clinical studies. We varied the concentration of nicotine during the alcohol abstinence week to investigate the effect of concentration on consumption. In Experiment 2A, the concentration of nicotine was maintained at 30 $\mu\text{g/mL}$ during the alcohol abstinence week, and we found that nicotine consumption increased in both sexes when alcohol was not available. When alcohol was re-introduced, both alcohol

and nicotine consumption returned to levels similar to those seen prior to alcohol abstinence. However, when the nicotine concentration was lowered to 20 µg/mL during alcohol abstinence, we found that nicotine consumption, but not preference, significantly decreased for both sexes. When alcohol was re-introduced after one week of abstinence, there was a re-instatement of alcohol consumption to levels that were significantly higher compared with pre-abstinent levels. This suggests that there was a rebound increase in alcohol consumption after abstinence that did not occur when the nicotine concentration was maintained at 30 µg/mL. We speculate that 20 µg/mL of nicotine may not be rewarding enough to maintain consumption, particularly since there was very little consumption of the 15 µg/mL nicotine solution, and that overall drug abstinence may be occurring during the alcohol abstinence period. However, when nicotine was maintained at 30 µg/mL, a concentration that elicits higher consumption, we speculate that the absence of alcohol prompted a compensatory increase in consumption of this more rewarding concentration of nicotine. Thus, with these two experiments, we were able to model a compensatory increase in nicotine consumption as well as a re-instatement of alcohol consumption after abstinence. Future work will take advantage of these models to investigate drugs or genetic manipulations that could help reduce nicotine consumption and alcohol re-instatement.

Intermittent drug consumption is defined by non-daily consumption. Non-daily alcohol drinking such as repeated binge drinking has been modeled in mice and rats using chronic intermittent alcohol consumption procedures, which result in high levels of alcohol consumption (Wise, 1973; Simms et al., 2008) that can exceed levels achieved in continuous access procedures (Hwa et al., 2011). The characteristics of and mechanisms underlying intermittent, non-daily smoking have been vastly understudied, and this population of smokers is increasing over time (Pulvers et al., 2015). Importantly, the smoking patterns of intermittent, non-daily smokers are more strongly associated with alcohol, such as smoking in a bar environment or while drinking alcohol, compared with daily smokers (Shiffman et al., 2014). Chronic, intermittent access to nicotine also elevates consumption compared with continuous access in rats (Cohen et al., 2012), similar to intermittent access to alcohol. However, there are no animal models of intermittent nicotine and alcohol co-consumption in mice or rats, thus we investigated the effect of intermittent access to alcohol and nicotine in male and female mice. We found that average daily alcohol consumption, but not nicotine consumption, was higher in our 3-bottle intermittent access test compared with our 3-bottle continuous access test for male mice. Female mice showed a very strong trend for higher alcohol consumption during the intermittent access test. These data suggest that combined intermittency elevates alcohol but not nicotine consumption. Intermittent drug access can result in behavioral changes in addition to the escalation of consumption. For example, the development of quinine-resistant alcohol consumption is observed after chronic intermittent access alcohol consumption but not continuous access alcohol consumption (Hopf et al., 2010). These behavioral changes likely stem from the recruitment of molecular mechanisms that are uniquely associated with the repeated drug intake and withdrawal that occurs during intermittent access consumption. For example, intermittent alcohol consumption increases, but continuous alcohol consumption decreases, the levels of protein kinase M zeta in the ventral striatum of mice (Lee et al., 2014). It is possible that during combined intermittent access, alcohol recruits molecular pathways that result in elevated intake, but nicotine does

not, resulting in an elevation of alcohol, but not nicotine consumption, in the intermittent access test. Although the rates at which the alcohol and nicotine concentrations were ramped up were different in each of our experiments, we do not expect this to be a confounding factor in the amount of drug intake, as in our experience, altering the ramp rate does not affect the average levels of drug consumption achieved.

Physical dependence to alcohol or nicotine in mice is often induced by forced consumption (Bhutada et al., 2010), forced exposure (Damaj et al., 2003; Metten et al., 2010), inhibition of drug metabolism (Perez et al., 2015) or a combination of the above. Although these procedures are excellent at inducing physical dependence, they induce dependence in a non-voluntary manner and may not be as clinically relevant compared to models that induce dependence through self-administration. Although the percent preference for single-drug alcohol is usually higher than 50% depending on the alcohol concentration, the percent preference for single-drug nicotine consumption is usually below 50% for most concentrations. In our co-consumption models, we observed percent preferences that were above 50% for alcohol and nicotine combined, and we assessed whether our chronic intermittent co-consumption test resulted in physical dependence. We found that after four weeks of intermittent co-consumption of alcohol and nicotine, male and female mice showed somatic signs of withdrawal, indicating that the animals developed physical dependence. The withdrawal signs that were observed after co-consumption were different between males and females, illustrating a sex difference in withdrawal from co-consumption. Chronic, voluntary single-drug nicotine consumption (Locklear et al., 2012) and single-drug alcohol consumption (Hwa et al., 2011) can also result in physical dependence in mice. In mice that are co-injected with alcohol and nicotine, simultaneous withdrawal from both drugs produces more severe withdrawal signs compared with single drug withdrawal (Perez et al., 2015). Although we cannot measure the proportion of withdrawal symptoms that are due to alcohol or nicotine in our co-consumption procedure, it is likely that both drugs are contributing to the withdrawal signs. Our data demonstrate that intermittent, oral co-consumption of alcohol and nicotine results in significant physical dependence, and would be a useful model to test genetic manipulations or drugs that may reduce consumption and subsequent withdrawal signs that may be helpful in patients concurrently addicted to both drugs.

5. CONCLUSIONS

We found that male and female C57BL/6 mice readily consumed alcohol and nicotine in voluntary, oral co-consumption procedures without the use of sweeteners. These procedures can capture interactions between alcohol and nicotine that occur during co-consumption, and have greater clinical relevance to human co-morbid alcohol and nicotine addiction compared with procedures that use investigator-administered drugs. Our key findings were that co-consumption of alcohol and nicotine resulted in lower levels of alcohol intake compared with single-drug alcohol consumption tests in males. Alcohol consumption was not affected by nicotine availability in female mice. The concentration of nicotine influenced nicotine consumption levels during an alcohol abstinence phase, as well as alcohol consumption levels when alcohol was re-introduced. Finally, we investigated intermittency in a co-consumption model and found that compared with a continuous access procedure,

intermittent access to alcohol and nicotine resulted in higher alcohol consumption, and trended towards decreased nicotine consumption. Intermittent access co-consumption also resulted in significant somatic withdrawal signs, indicating that voluntary consumption resulted in physical dependence in mice.

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Figure 1.

Experiment 1: Alcohol and nicotine consumption and preference in sequential alcohol and nicotine two-bottle choice tests in male and female C57BL/6 and DBA/2J mice. A) Female C57BL/6 mice consume more alcohol compared with male C57BL/6 mice at the 10 and 14% alcohol concentrations. Male DBA/2J mice consume more alcohol at the 6 and 20% concentrations compared with female DBA/2J mice. B) Male DBA/2J mice have higher alcohol preference compared with female DBA/2J mice at the 6% alcohol concentration. C) Female C57BL/6 mice have higher average daily nicotine consumption at the 40 and 50 $\mu\text{g/mL}$ concentrations, D) and have higher nicotine preference at the 50 $\mu\text{g/mL}$ concentration compared with male C57BL/6 mice. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$ compared between sexes within a strain using Sidak's multiple comparison post-hoc test. Male C57BL/6 $n = 10$, female C57BL/6 $n = 10$, male DBA/2J $n = 10$, female DBA/2J $n = 10$.

Figure 2.

Experiment 2A: Alcohol and nicotine co-consumption in a continuous access 3-bottle choice test in male and female C57BL/6 mice. The average daily alcohol and nicotine consumption levels in A) male and C) female mice. Alcohol, nicotine and water preference for B) male and D) female mice. The nicotine concentration was maintained at 30 μg/mL during the alcohol abstinence week. Average daily nicotine consumption and preference increased during the alcohol abstinence week in both males and females. ** $P < 0.01$, **** $P < 0.0001$ for an overall effect of week in a two-way repeated measures ANOVA. Male C57BL/6 $n = 15$, female C57BL/6 $n = 15$.

Figure 3.

Experiment 2B: Alcohol and nicotine co-consumption in a continuous access 3-bottle choice test in male and female C57BL/6 mice. The average daily alcohol and nicotine consumption levels in A) male and C) female mice. Alcohol, nicotine and water preference for B) male and D) female mice. The nicotine concentration was decreased to 20 µg/mL during the alcohol abstinence week. Average daily alcohol consumption increased when alcohol was re-introduced for both males and females. Nicotine consumption decreased during the week of alcohol abstinence in both sexes. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$ for an overall effect of week in a two-way repeated measures ANOVA. Male C57BL/6 $n = 15$, female C57BL/6 $n = 15$.

Figure 4.

A) In male C57BL/6 mice, the average daily alcohol consumption was lower for the 10 and 20% concentrations in the continuous access co-consumption 3-bottle choice tests compared with the sequential, continuous access 2-bottle choice tests. **** $P < 0.0001$ using a Sidak's multiple comparison post-hoc test. B) Female C57BL/6 mice showed similar levels of average daily alcohol consumption in both the 3-bottle choice test and the sequential 2-bottle choice test. There was no difference in the average daily consumption for the 30 $\mu\text{g/mL}$ nicotine concentration between the 3-bottle choice test and the 2-bottle choice test for C) males or D) females. Continuous access 3-bottle choice test: male C57BL/6 $n=28-30$,

female C57BL/6 $n=29-30$. Continuous access 2-bottle choice test: male C57BL/6 $n=10$, female C57BL/6 $n=10$.

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Figure 5.

Experiment 3: Alcohol and nicotine co-consumption in an intermittent 3-bottle choice test. During week 1, mice received 3% alcohol and 5 μ g/mL nicotine on Monday, 6% alcohol and 10 μ g/mL nicotine on Wednesday, and 10% alcohol and 15 μ g/mL nicotine on Friday. During weeks 2–4, mice received 20% alcohol and 30 μ g/mL nicotine every Monday, Wednesday and Friday. The average daily alcohol and nicotine consumption levels in A) male and C) female mice. Alcohol, nicotine and water preference for B) male and D) female mice. Male C57BL/6 $n=15$, female C57BL/6 $n=15$.

Figure 6.

Withdrawal scores for male ($n=15$) and female ($n=15$) C57BL/6 mice after the intermittent alcohol and nicotine co-consumption test. * $P<0.05$, ** $P<0.01$ in a one-sample, two-way Student's t-test.

Figure 7.

The average daily alcohol consumption for A) male and B) female mice during the intermittent access 3-bottle choice test (I) compared with the continuous access 3-bottle choice test (C). ** $P < 0.01$ using a Student's t-test. The average daily nicotine consumption for the 30 $\mu\text{g/mL}$ concentration in C) males or D) females between the continuous and intermittent access 3-bottle choice tests. Continuous access 3-bottle choice tests: male and female C57BL/6 $n=28-30$ each. Intermittent access 3-bottle choice test: male and female C57BL/6 $n=15$ each.