Unequal interactions between alcohol and nicotine co-consumption: Suppression and enhancement of concurrent drug intake

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Abstract

Background: Alcohol and nicotine addiction are prevalent conditions that often co-occur. Despite the prevalence of alcohol and nicotine co-use, factors that influence the suppression and enhancement of concurrent alcohol and nicotine intake are largely unknown.

Aims: Our goals were to assess how nicotine abstinence and availability influenced concurrent alcohol consumption, and to determine the impact of quinine adulteration of alcohol on aversion resistant alcohol consumption and concurrent nicotine consumption.

Methods: Male and female C57BL/6J mice voluntarily consumed unsweetened alcohol, nicotine and water in a chronic 3-bottle choice procedure. In Experiment 1, nicotine access was removed for 1 week and re-introduced the following week, while the alcohol and water bottles remained available at all times. In Experiment 2, quinine (100-1000 μM) was added to the 20% alcohol bottle, while the nicotine and water bottles remained unaltered.

Results: In Experiment 1, we found that alcohol consumption and preference were unaffected by the presence or absence of nicotine access in both male and female mice. In Experiment 2, we found that quinine temporarily suppressed alcohol intake and enhanced nicotine, but not water, preference in both male and female mice. Chronic quinine suppression of alcohol intake increased nicotine consumption and preference in female mice, and increased water preference in male mice.

Conclusions: Our results highlight the complex interactions and sex differences between voluntarily consumed alcohol and nicotine. Understanding the behavioral and molecular factors that mediate these interactions will provide further insight into the nature of alcohol and nicotine co-use.

Keywords

Alcohol, nicotine, quinine, addiction, aversion

Introduction

Alcohol and nicotine are the two most commonly abused addictive drugs and the majority of alcohol dependent individuals are also dependent on nicotine (Batel et al., 1995; Falk et al., 2006; Miller and Gold, 1998). Persons co-dependent on alcohol and nicotine have more severe drug dependence symptoms such as greater craving and withdrawal signs, higher drug consumption, increased difficulty maintaining abstinence, and have higher mortality compared with persons dependent on alcohol or nicotine alone (Heffner et al., 2011; Hurt et al., 1996; King et al., 2009; Leeman et al., 2008; Marks et al., 1997). Despite the high prevalence and increased health consequences of alcohol and nicotine co-dependence, there are currently no FDA-approved drugs for the treatment of alcohol and nicotine co-dependence. Several reviews have highlighted key knowledge gaps that have limited the development of new drugs, such as the need to better understand the neurobiology of co-dependence, the need for identification of drug targets that mediate both alcohol and nicotine dependence, and the need to develop a greater variety of animal models that reflect alcohol and nicotine co-dependence (Tarren and Bartlett, 2017; Van Skike et al., 2016).

Animal models that allow for the voluntary, separate consumption of alcohol and nicotine are not commonly used, thus the molecular and behavioral interactions in the voluntary intake of alcohol and nicotine are not well understood. A limited number of studies in rats have used intravenous self-administration of nicotine in combination with operant oral consumption of alcohol (Lê et al., 2010; Lê et al., 2014) or with alcohol consumption in a 2-bottle choice procedure (Maggio et al., 2018). Both sets of procedures involve significant training as well as technical and surgical requirements. In contrast, 2-bottle choice studies are frequently used in rodents to assess alcohol or nicotine consumption (Lee and Messing, 2011; Lee et al., 2014; Locklear et al., 2012; Meliska et al., 1995; Powers et al., 2013; Simms et al., 2008). In these studies, the animals are individually housed with two fluid bottles, one containing drug and one water, and the animals are free to consume from both bottles. These bottle choice studies are less technically challenging and are more high-throughput compared with operant administration studies.

We have previously developed a novel 3-bottle choice co-consumption model where mice voluntarily consume unsweetened alcohol, unsweetened nicotine, and water from 3 separate drinking bottles (O'Rourke et al., 2016; Touchette et al., 2018). Using this model, we investigated the effects of forced alcohol abstinence and intermittent drug access, and the impact of pre-clinical drug treatment on concurrent alcohol and nicotine consumption in male and female C57BL/6J mice (O'Rourke et al., 2016; Touchette et al., 2018). We reported that after 3 weeks of chronically co-consuming alcohol and nicotine, forced alcohol abstinence resulted in an increase in concurrent nicotine consumption and preference in male and female C57BL/6J mice, suggesting that the mice compensated for the absence of alcohol by increasing their consumption of nicotine (O'Rourke et al., 2016). In the current study, one of our goals was to determine whether forced nicotine abstinence influenced concurrent alcohol consumption in the same manner.

A prominent feature of alcohol use disorders (AUDs) in humans is consumption of alcohol despite adverse legal, health, economic, and societal consequences. The continued consumption of alcohol despite the addition of the bitter tastant quinine has been frequently used as a model of compulsive alcohol intake or aversion-resistant alcohol consumption in both rats and mice (Hopf et al., 2010; Hopf and Lesscher, 2014; Lei et al., 2016b; Sneddon et al., 2019; Spanagel et al., 1996). Quinine suppression of alcohol consumption has not yet been evaluated in a voluntary alcohol and nicotine co-consumption model. Here, we investigate the impact of acute and chronic addition of quinine to the alcohol bottle on concurrent nicotine consumption.

Overall, we find an unequal interaction between alcohol and nicotine, where alcohol consumption is unaffected by the presence or absence of nicotine access. In contrast, the addition of quinine temporarily suppressed alcohol preference and enhanced concurrent nicotine preference in both sexes. Chronic suppression of alcohol consumption with quinine produced a long-term enhancement of concurrent nicotine consumption in female, but not male, C57BL/6J mice.

Materials and Methods

Animals and Reagents

12 male and 12 female C57BL/6J mice from The Jackson Laboratory (Sacramento, CA) acclimated to our facility for six days before beginning behavioral experiments at 55 days old. All mice underwent both experimental procedures. Mice were group housed in standard cages under a 12-h light/dark cycle until the start of behavioral experiments, after which they were individually housed. 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.

Alcohol (ethanol) (Decon Labs, King of Prussia, PA), and nicotine tartrate salt (Acros Organics, Thermo Fisher Scientific, Chicago, IL) were mixed with tap water to the concentrations reported for each experiment. The concentrations of nicotine are reported as free base, and nicotine solutions were not filtered or pH adjusted. The alcohol, nicotine and water bottles were unsweetened at all times. Quinine hydrochloride (Spectrum Chemical Manufacturing Corp, Gardena, CA) was added to the 20% alcohol bottle at concentrations of 100, 200, 500 and 1000 μM in Experiment 2.

Experiment 1: Effects of nicotine abstinence on concurrent alcohol consumption.

Mice were singly housed in custom cages that accommodated three drinking bottles (Ancare, Bellmore, NY) containing water, nicotine or alcohol at different concentrations. The concentrations for the first week consisted of 3% alcohol (v/v) in one bottle, 5 μg/mL nicotine in the second bottle and water in the third bottle. The concentrations for the second week were 10% alcohol and 15 μg/mL nicotine, and were 20% alcohol and 30 μg/mL nicotine for the third week. During the fourth week, the nicotine bottle was removed and the 20% alcohol and water bottles remained available. During the fifth week, all three

bottles were again presented at the 20% alcohol and 30 μg/mL nicotine concentrations. Food was freely available and the mice were not fluid restricted at any time. The bottles were weighed every other day and the solutions refreshed every 3-4 days. The positions of the bottles were alternated after weighing to account for side preferences. Mice were weighed once a week. Fluid evaporation and potential dripping were accounted for by the presence of a set of alcohol, nicotine and water bottles on an empty control cage. The weight of fluid loss from these bottles was subtracted from all bottle weights throughout the study.

Experiment 2: The impact of quinine adulteration on concurrent alcohol and nicotine consumption.

Immediately after completion of Experiment 1, the mice proceeded to Experiment 2 without a break in alcohol or nicotine consumption. Quinine (100 μM) was added to the 20% alcohol bottle for 3 days (acute phase), while the water and 30 μg/mL nicotine bottles remained unaltered. After 3 days of 100 μM quinine, the quinine concentration in the 20% alcohol bottle was increased to 200, 500 and 1000 μM, with each concentration presented for 6 days (chronic phase). The bottles were weighed and rotated every day and the mice were weighed once a week. Fluid evaporation and bottle dripping were controlled for by the presence of a set of bottles on an empty control cage. The weight of fluid loss from these bottles was subtracted from all bottles throughout the study.

Statistical Analysis

The average daily alcohol (g/kg) and nicotine consumption (mg/kg) for each drug concentration were calculated based on the weight of the fluid consumed from the bottles, the density of the solution (for alcohol only), and the weight of the individual mouse. The percent preference for alcohol, nicotine, and water bottles was calculated as the weight of the fluid consumed from the bottle, divided by the summed

weight of fluid consumed from all three bottles, multiplied by 100. In Experiment 2, the average daily drug consumption and preference at the 100 μM quinine concentration was calculated across the 3 days of 100 μM quinine presentation, and across 6 days for all other quinine concentrations. Data from male and female mice were analyzed separately as female mice consume more drug compared with male mice (O'Rourke et al., 2016). All analyses were calculated using Prism 8.0 (GraphPad, La Jolla, CA). Data were tested for normality and variance. Comparison within groups was analyzed using one-way ANOVA followed by Dunnett's multiple comparisons tests.

Results

Experiment 1: Effect of forced nicotine abstinence on concurrent alcohol consumption.

A schematic outline of our experiments is shown in **Figure 1**. In Experiment 1, we evaluated the effect of forced nicotine abstinence on concurrent alcohol consumption and preference. Male and female C57BL/6J mice had continuous access to alcohol (v/v), nicotine (μg/mL) and water for 3 weeks in a 3-bottle choice procedure, with drug concentrations of 3% alcohol and 5 μg/mL nicotine presented during week 1, 10% alcohol and 15 μg/mL nicotine during week 2 and 20% alcohol and 30 μg/mL nicotine presented during week 3. During week 4, the nicotine bottle was removed and mice had access to the 20% alcohol and water bottles. In male mice, there was no change in the average alcohol consumption compared with the previous week (week 3) or after nicotine bottle was re-introduced (week 5) (**Figure 2A**, $F(2,22)=1.49$, $P=0.25$). Female mice consumed more alcohol compared with male mice but a similar pattern of alcohol consumption was observed when the nicotine bottle was removed and re-introduced (**Figure 2B**, $F(2,22)=0.3946$, $P=0.63$). Removal of the nicotine bottle during week 4 increased preference for the water bottle in male mice (**Figure 2C**, $F(2, 22)=75.7$, $P<0.0001$). A similar increase in water

preference was observed in female mice during the forced nicotine abstinence week (**Figure 2D**, F(2,22)=41.6, *P*<0.0001). No significant changes in preference for the alcohol or nicotine bottles were observed across weeks 3 to 5 in the male or female mice.

Experiment 2: The impact of quinine adulteration on concurrent alcohol and nicotine consumption.

We tested the effect of quinine adulteration of alcohol on concurrent alcohol and nicotine intake in male and female C57BL/6J mice. Quinine (100 μM) was added to the 20% alcohol bottle for 3 days. In male mice, 100 μM quinine suppressed alcohol consumption on Days 1 and 2 (**Figure 3A**, F(3,33)=15.83, *P*<0.0001) and suppressed alcohol preference on Day 1 compared with alcohol consumption without quinine (**Figure 3C**, F(3,33)=15.20, *P*<0.0001). In female mice, 100 μM quinine adulteration of alcohol suppressed alcohol consumption (**Figure 3B**, F(3,33)=22.16, *P*<0.0001) and alcohol preference on Day 1 (**Figure 3D**, F(3,33)=22.57, *P*<0.0001). Quinine suppression of alcohol consumption was temporary, as male mice overcame the 100 μM quinine suppression by Day 3, and female mice by Day 2.

The addition of 100 μM quinine to the 20% alcohol bottle produced a temporary increase in concurrent nicotine preference. In both male and female mice, preference for the nicotine bottle increased on Day 1 and returned to baseline levels on Day 2 (**Figure 3C**, males F(3,33)=7.243, *P*=0.001; **Figure 3D**, females F(3,33)=10.03, *P*<0.001). No significant changes in water preference was observed at any time for either sex.

To determine the effect of chronic quinine adulteration of alcohol consumption on concurrent nicotine intake, the quinine concentration in the 20% alcohol bottle was increased to 200, 500 and 1000 μM, with each concentration presented for 6 days. We found that the average daily alcohol consumption and preference was significantly suppressed at 200, 500 and 1000 μM in male mice (**Figure 4A** consumption: F(4,44)=25.05, *P*<0.0001; **Figure 4C** preference F(4,44)=25.05, *P*<0.0001). In female

mice, quinine suppressed the average daily alcohol consumption and preference at all concentrations (**Figure 4B** consumption: $F(4,44)=25.33, P<0.0001$; **Figure 4D** preference: $F(4,44)=27.98, P<0.0001$).

Chronic suppression of alcohol consumption with quinine produced an increase in nicotine consumption and preference in female mice. During presentation of 20% alcohol+1000 μM quinine, the average daily nicotine consumption was significantly higher compared with consumption during the 100 and 500 μM quinine concentrations (**Figure 4B**, $F(4,44)=7.827, P=0.005$). Preference for the nicotine bottle was significantly greater during the 100 and 1000 μM quinine+alcohol presentation compared with no quinine (**Figure 4D**, $F(4,44)=14.12, P<0.0001$). Female mice showed no change in preference for the water bottle at any time. In male mice, chronic suppression of alcohol consumption with 500 μM quinine resulted in an increase in preference for the water bottle and had no effect on nicotine consumption or preference (**Figure 4C**). During the 1000 μM quinine+alcohol presentation, both water and nicotine preference was significantly increased compared with no quinine (**Figure 4C**, water preference: $F(4,44)=7.152, P=0.002$; nicotine preference: $F(4,44)=3.148, P=0.048$). There was no significant increase in nicotine consumption at any time. These data show that chronic alcohol adulteration with quinine produces an increase in nicotine consumption and preference without affecting water preference in female mice, but primarily increases water, and not nicotine, intake in male mice.

Discussion

In this study, we used a 3-bottle choice procedure that allows for voluntary, chronic co-consumption of alcohol and nicotine to investigate the suppression and enhancement of concurrent drug intake in male and female C57BL/6J mice. We found that alcohol consumption and preference is unaffected by forced nicotine abstinence or the re-introduction of nicotine access in both male and female C57BL/6J mice. Our previous study using this same 3-bottle choice model showed that forced alcohol abstinence produced an

enhancement in concurrent nicotine consumption and preference in male and female C57BL/6J mice (O'Rourke et al., 2016). Together, our work shows a complex, unequal interaction between alcohol and nicotine intake, where alcohol intake is unresponsive to the presence or absence of the nicotine bottle, but nicotine consumption and preference is influenced by the presence or absence of alcohol consumption.

Investigating the interactions between voluntary alcohol and nicotine intake in animal models can help to inform complex human data on alcohol and nicotine co-use. The majority of tobacco smokers also use alcohol, yet smoking cessation trials frequently incorporate alcohol-related exclusion criteria and do not track co-use of alcohol in their subjects (Leeman et al., 2007), thus data on the treatment of co-users are lacking. Nearly one quarter of smokers report hazardous alcohol consumption patterns as defined by the NIAAA (Toll et al., 2012). These individuals have lower smoking cessation rates compared with moderate alcohol drinkers, highlighting that some co-users are less likely to successfully quit alcohol and nicotine use compared with other co-users (Toll et al., 2012). The behavioral and molecular interactions between different types of alcohol and nicotine co-use need to be better understood to facilitate the development of successful therapies. Treatment of both alcohol and nicotine dependence simultaneously with one pharmacotherapy has shown limited success in the clinic. Naltrexone and acamprosate, two approved drugs for the treatment of alcohol dependence, do not have any impact on cigarette smoking (Kahler et al., 2017; Fucito et al., 2012). Varenicline is an $\alpha4\beta2$ nicotinic acetylcholine receptor (nAChR) partial agonist approved for smoking cessation (Rollema et al., 2010). A recent animal study examining voluntary alcohol and nicotine co-consumption in alcohol-preferring female rats shows that varenicline reduces nicotine intake but has no effect on concurrent alcohol intake (Maggio et al., 2018), suggesting that monotherapy with varenicline may be ineffective for alcohol cessation treatment in some populations. Indeed, varenicline has shown mixed results in reducing alcohol consumption in human studies (de Bejczy et al., 2015; Litten et al., 2013; Mitchell et al., 2012; O'Malley et al., 2018; Plebani et al., 2013). Dual pharmacological treatment, such as combining naltrexone with nicotine replacement therapy, has been incorporated in trials to enhance the likelihood of successful alcohol and smoking abstinence

(Kahler et al., 2017; Toll et al., 2010). Optimizing dual treatment strategies for different patterns of alcohol and nicotine co-use may provide further benefits.

One characteristic of AUDs is the continued consumption of alcohol despite adverse consequences. Quinine adulteration of alcohol consumption has been frequently used to model aversion-resistant alcohol intake (Hopf et al., 2010; Hopf and Lesscher, 2014; Lei et al., 2016b; Sneddon et al., 2019; Spanagel et al., 1996). Quinine adulteration of alcohol consumption had not been previously examined in conjunction with concurrent nicotine consumption. In this study, we found that addition of 100 μM quinine to the alcohol bottle suppressed alcohol consumption and preference in both male and female mice on the first day it was presented (**Figure 3**). Interestingly, the suppression of alcohol intake was associated with increased preference for the nicotine bottle and not the water bottle (**Figure 3**). These data suggest that the mice compensate for the reduction in quinine-adulterated alcohol intake by selectively increasing preference for the unsweetened nicotine bottle, even though the water bottle is readily available. These results support our previous findings that nicotine consumption and preference is enhanced during forced alcohol abstinence (O'Rourke et al., 2016), and show that suppression of alcohol consumption, either by removing access to the alcohol bottle or reducing the palatability of alcohol, produces an enhancement of concurrent nicotine consumption.

The suppression of alcohol intake with 100 μM quinine was temporary, as female mice overcame the aversion by Day 2 and males by Day 3. This suggests that mice chronically co-consuming alcohol and nicotine can overcome quinine adulteration of alcohol in a few days. Other recently published studies also show that C57BL/6J mice quickly develop quinine-resistant alcohol consumption. For example, Lesscher and colleagues (2010) show that male C57BL/6J mice are resistant to quinine adulteration of alcohol after 2 weeks of intermittent alcohol consumption (Lesscher et al., 2010), whereas Lei and colleagues (2016) show that a single session of unadulterated alcohol consumption is sufficient to produce aversion-resistant alcohol consumption (Lei et al., 2016b). In contrast, prior studies in rats have required a minimum of 3

months of intermittent alcohol consumption before the development of aversion resistant alcohol consumption (Hopf et al., 2010; Spanagel et al., 1996).

We increased the concentration of quinine in the alcohol bottle to determine the effect of long-term suppression of alcohol consumption on concurrent nicotine intake. We found that alcohol consumption was suppressed in a concentration-dependent manner in both male and female mice (**Figure 4**). Associated with the decrease in alcohol preference, male mice showed increased preference for the water bottle at 500 μM quinine with no significant changes in nicotine consumption or preference, suggesting that male mice were compensating for the long-term suppression of alcohol intake by increasing their water preference. Only at the highest quinine concentration did male mice show increased preference for the nicotine bottle. In contrast, female mice showed increased nicotine consumption and preference as the quinine concentration increased, and did not show any increase in water preference for the duration of the study. Our data suggest that female mice compensate for the long-term suppression of alcohol intake by increasing their nicotine intake. These data illustrate an important sex difference in compensatory drug consumption and highlight the need to continue investigating both sexes to identify important differences that can influence addiction-related behaviors and potential treatment strategies. Nearly all of the prior research on aversion-resistant drinking has focused on male animals. One recent study that investigates aversion-resistant drinking in male and female C57BL/6J mice shows no sex difference in the level of quinine suppressed alcohol consumption, even though female mice consume more alcohol than males (Sneddon et al., 2019).

Alcohol and nicotine addiction are heritable disorders that share common genetic factors (Swan et al., 1996; Swan et al., 1997; True et al., 1999). The nAChRs are the primary target of nicotine, whereas alcohol acts on many different receptor systems. Alcohol is not a direct agonist at the nAChRs but can modulate nAChR activity (Borghese et al., 2003). Voluntary alcohol consumption in rats increases the release of acetylcholine (ACh, the endogenous ligand for nAChRs), in the ventral tegmental area, a reward-related brain region (Larsson et al., 2005). In the brain, the nAChRs are composed of 5 out of 11 potential subunits (α2-7, α9-10, β2-4) and of these subunits, the α4 and α6 subunits are most heavily

implicated in mediating alcohol- and nicotine-related behaviors (Hendrickson et al., 2013). We recently reported that sazetidine-A, a pre-clinical drug that acts on nAChRs containing the α4, α6 or α7 subunits, reduces alcohol consumption but not concurrent nicotine consumption in male and female mice (Touchette et al., 2018). The contribution of nAChRs to aversion-resistant alcohol consumption has only recently been investigated. Leão and colleagues (2015) show that after male rats are made alcohol dependent, daily nicotine injections produce more aversion-resistant alcohol consumption compared with daily saline injections, and this effect is blocked by mecamylamine, a non-specific nAChR antagonist (Leão et al., 2015). To our knowledge, this is the only study investigating nAChR effects in aversion-resistant drinking, and their data suggest that activation of nAChRs may facilitate compulsive alcohol consumption. Which nAChR subunits are involved in this enhancement of aversion-resistant alcohol consumption and where the receptors are located remains to be determined. In addition, the roles of other receptor systems implicated in mediating aversion-resistant drinking, such as the orexin-1 and NMDA receptors (Lei et al., 2016a; Seif et al., 2015) have not yet been investigated in models of quinine adulterated alcohol and nicotine co-use.

In summary, our data highlight a complex interaction between alcohol and nicotine co-consumption. We found that the presence or absence of nicotine access does not affect alcohol consumption. However, adulterating alcohol with quinine produced a temporary suppression of alcohol consumption and preference, which was associated with an enhancement of concurrent nicotine preference. Chronic suppression of alcohol consumption with quinine produced a sex difference in compensatory nicotine consumption, where female mice showed a greater enhancement of nicotine consumption and preference compared with male mice. Further behavioral and molecular dissection of these interactions will provide a better understanding of the neurobiology underlying alcohol and nicotine co-use.

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Figure legends

Figure 1. *Schematic of experimental procedures.* Unsweetened alcohol % (A, v/v), nicotine (N, μg/mL) and water were presented in a 3-bottle choice consumption procedure. Quinine (Q, μM) was added to the 20% alcohol bottle in Experiment 2.

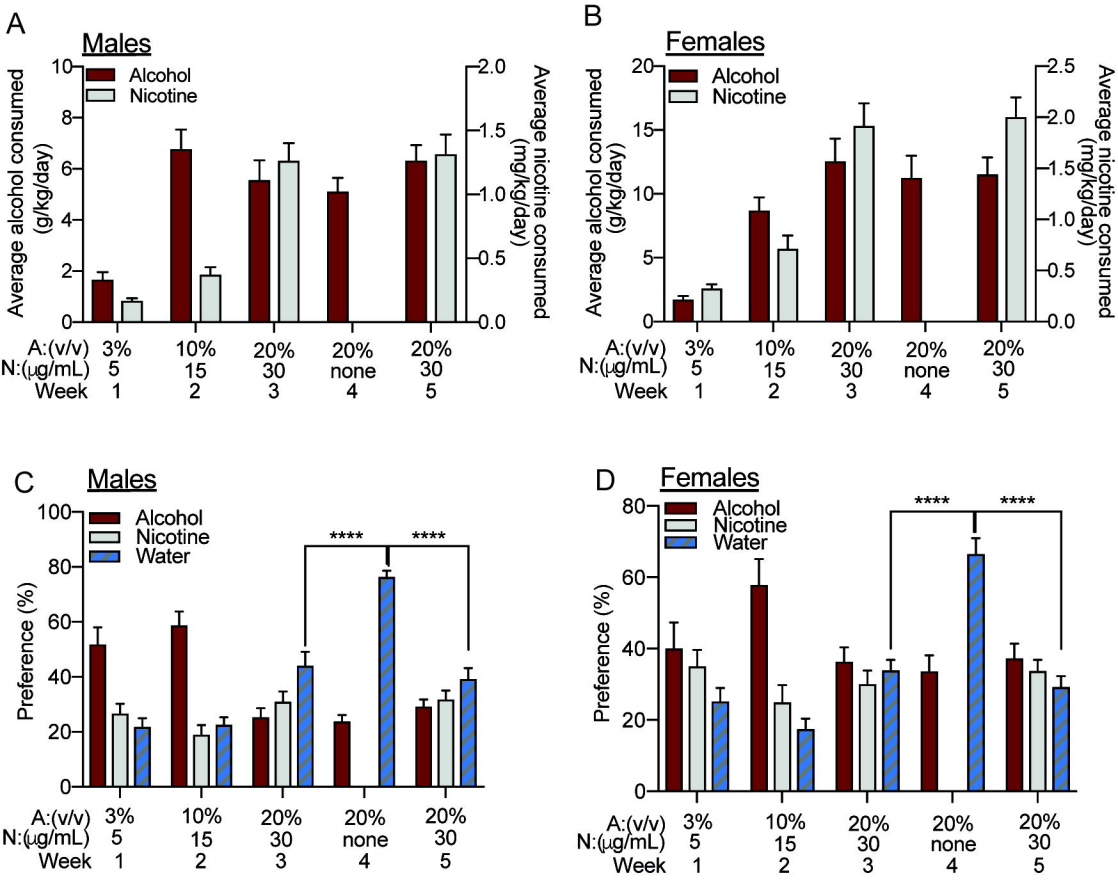
Figure 2. *Alcohol consumption and preference is unaffected by the absence or re-introduction of concurrent nicotine access.* Average alcohol (g/kg) and nicotine (mg/kg) consumption in (A) male and (B) female mice. Removal of the nicotine bottle during week 4 increased the preference for the water bottle but not the alcohol bottle in (C) male and (D) female mice. ****P<0.0001 by a Tukey's multiple comparisons test. n=12 per sex, mean ± SEM.

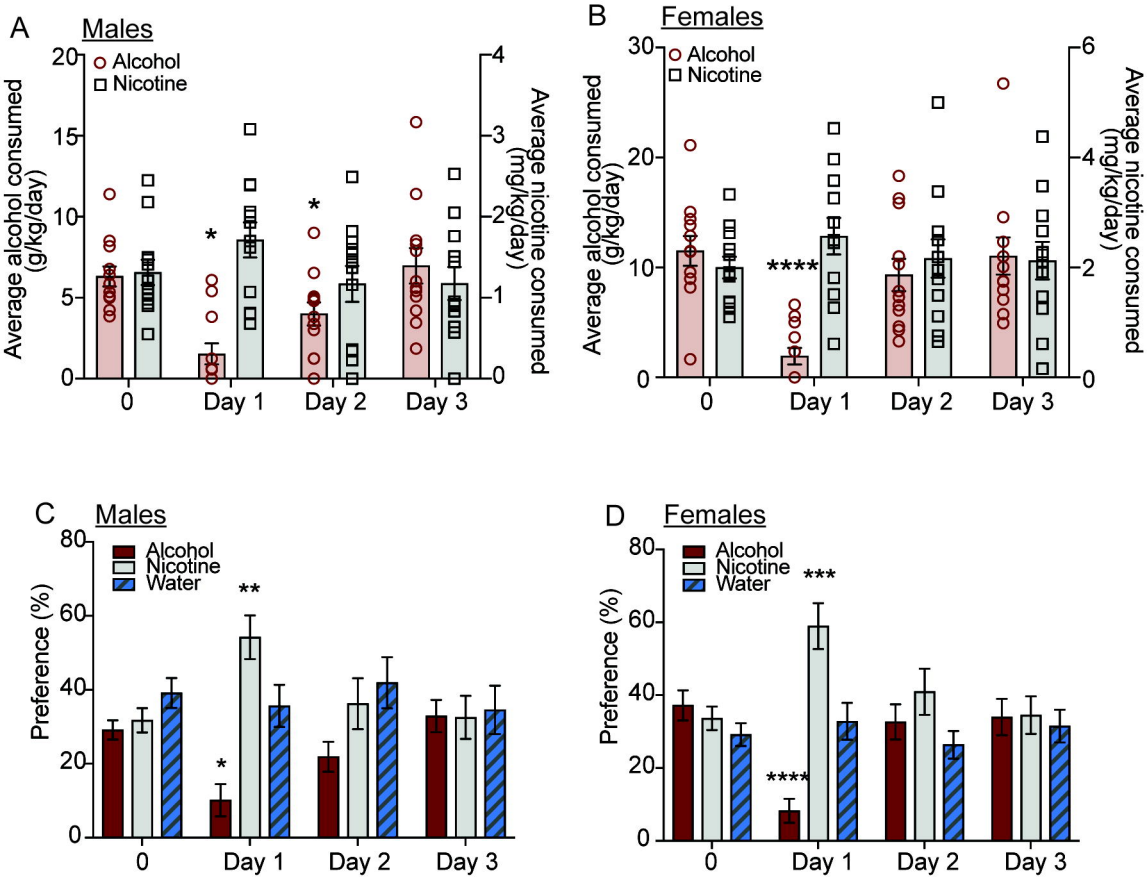
Figure 3. *Temporary quinine-induced suppression of alcohol intake produces an increase in concurrent nicotine preference.* Addition of 100 μM quinine to the 20% alcohol bottle occurred on Days 1-3. Quinine suppressed alcohol consumption on Day 1 in male (A) and female (B) mice compared to the average alcohol consumption without quinine (0 bar). Quinine suppressed preference for the alcohol bottle and increased concurrent preference for the nicotine bottle in both (C) male and (D) female mice on Day 1. *P<0.05, **P<0.01, ****P<0.0001 compared with the 0 quinine level within drug by a Dunnett's multiple comparisons test. n=12 per sex, mean ± SEM.

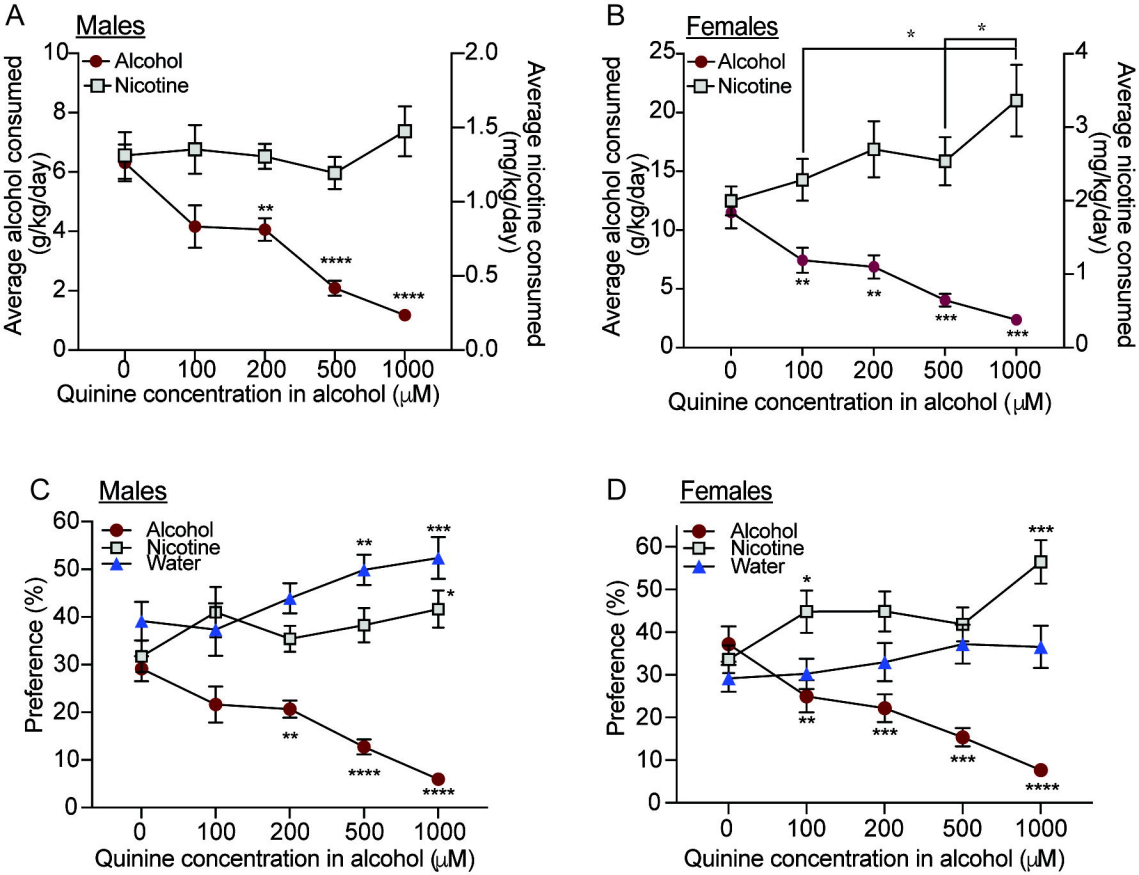
Figure 4. *Chronic suppression of alcohol intake by quinine increases nicotine consumption and preference.* (A) In male mice, the average daily consumption from the 20% alcohol bottle was suppressed by 200, 500 and 1000 μM quinine. (C) Male mice showed reduced preference for the alcohol bottle when 200, 500 and 1000 μM quinine was added. Male mice showed an increased preference for the water bottle when the alcohol bottle contained 500 and 1000 μM quinine and an increased preference for the nicotine

bottle when the alcohol bottle contained 1000 μM quinine. (B) In female mice, the average daily alcohol consumption was suppressed by all quinine concentrations, and the average daily nicotine consumption was increased when the alcohol bottle contained 1000 μM quinine. (D) Female mice showed a decrease in preference for the alcohol bottle at all quinine concentrations. Female mice showed an increase in nicotine preference when the alcohol bottle contained 100 and 1000 μM quinine. There were no changes in water preference at any quinine concentration in female mice. $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$ compared with 0 quinine levels of the same fluid, unless otherwise specified, by a Dunnett's multiple comparisons test. $n=12$ per sex, mean ± SEM.

	Expt 1					Expt 2			
Time	Week 1	Week 2	Week 3	Week 4	Week 5	3d	6d	6d	6d
A%:	3	10	20	20	20	20+100 Q	20+200 Q	20+500 Q	20+1000 Q
N μg/mL:	5	15	30		30	30	30	30	30
Water:	W	W	W	W	W	W	W	W	W


A
Males
Alcohol
Nicotine
Average alcohol consumed (g/kg/day)
Average nicotine consumed (mg/kg/day)
A:(v/v) 3% 10% 20% 20% 20%
N:(μg/mL) 5 15 30 none 30
Week 1 2 3 4 5
B
Females
Alcohol
Nicotine
Average alcohol consumed (g/kg/day)
Average nicotine consumed (mg/kg/day)
A:(v/v) 3% 10% 20% 20% 20%
N:(μg/mL) 5 15 30 none 30
Week 1 2 3 4 5
C
Males
Alcohol
Nicotine
Water
Preference (%)
****
****
A:(v/v) 3% 10% 20% 20% 20%
N:(μg/mL) 5 15 30 none 30
Week 1 2 3 4 5
D
Females
Alcohol
Nicotine
Water
Preference (%)
****
****
A:(v/v) 3% 10% 20% 20% 20%
N:(μg/mL) 5 15 30 none 30
Week 1 2 3 4 5


A
Males
Alcohol
Nicotine
Average alcohol consumed (g/kg/day)
Average nicotine consumed (mg/kg/day)
0
Day 1
Day 2
Day 3
B
Females
Alcohol
Nicotine
Average alcohol consumed (g/kg/day)
Average nicotine consumed (mg/kg/day)
C
Males
Alcohol
Nicotine
Water
Preference (%)
D
Females
Alcohol
Nicotine
Water
Preference (%)


A
Males
Alcohol
Nicotine
Average alcohol consumed (g/kg/day)
Average nicotine consumed (mg/kg/day)
Quinine concentration in alcohol (μM)
B
Females
Alcohol
Nicotine
Average alcohol consumed (g/kg/day)
Average nicotine consumed (mg/kg/day)
Quinine concentration in alcohol (μM)
C
Males
Alcohol
Nicotine
Water
Preference (%)
Quinine concentration in alcohol (μM)
D
Females
Alcohol
Nicotine
Water
Preference (%)
Quinine concentration in alcohol (μM)