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Research report

Genetic correlation between the free-choice oral consumption of nicotine and alcohol in C57BL/6J × C3H/HeJ F2 intercross mice

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Abstract

Previous studies in humans have demonstrated a high co-morbidity between alcoholism and smoking. This co-morbidity between alcohol and nicotine dependence can be attributed, in part, to common genetic factors. In rodents, behavioral and physiological responses to alcohol and nicotine also appear to share common genetic influences. In this report, the genetic correlation between free-choice oral nicotine and oral alcohol consumption was evaluated using an ascending two-bottle choice paradigm in C57BL/6 × C3H/HeJ F2 intercross mice. For all concentrations of nicotine (25, 50, and 100 μg/ml) and alcohol (3, 6, and 10%) tested, nicotine consumption was significantly correlated with alcohol consumption. Nicotine consumption at the highest nicotine concentration tested (100 μg/ml) showed low, but significant, correlations with the number of [^{3}H]-cytisine binding sites in the hippocampus ($r = 0.307$) and the number of [^{125}I]-α-bungarotoxin binding sites in the cortex ($r = -0.328$). No significant correlations between alcohol consumption and the number of either [^{3}H]-cytisine or [^{125}I]-α-bungarotoxin binding sites was observed. A polymorphism in the nicotinic receptor α4 subunit gene, *Chrna4*, showed a trend with nicotine consumption and a significant association with alcohol consumption in female but not male mice. These results indicate that common genetic factors influence nicotine and alcohol consumption in mice. However, neither individual differences in the expression of [^{3}H]-cytisine or [^{125}I]-α-bungarotoxin binding nicotinic receptors nor the polymorphism in *Chrna4* likely contribute to the genetic overlap that influences the consumption of both of these drugs of abuse in C57BL/6 × C3H/HeJ F2 mice.

Keywords: Co-morbidity; Preference; Nicotinic receptor; Polymorphism

1. Introduction

Studies in humans have demonstrated a high co-morbidity between alcoholism and smoking [3,21]. Approximately 80% of alcoholics are smokers while about 24% of the total adult population smoke cigarettes. Moreover, there is a direct correlation between the amount of alcohol consumed and the number of cigarettes consumed among alcoholics [2,9,22]. It also has been established that there is a considerable genetic influence on both alcoholism and smoking. Studies have estimated that approximately 50–60% of individual variation in either alcohol or tobacco consumption can be attributed to genetic factors [7,18–20,38] and a considerable amount of the genetic variance for alcoholism and smoking may be attributed to common genetic factors [43].

In rodents, it has been established that responses to alcohol and nicotine, the primary addictive agent in tobacco, also are significantly influenced by genetic factors. As is the case in humans, responses to alcohol and nicotine appear to share common genetic components. For example, several responses to nicotine and alcohol were correlated in a classic genetic cross between the selected mouse lines long-sleep (LS) and short-sleep (SS) [12]. In addition, the LS and SS mice, which

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were selected for individual differences in sensitivity to the sedative effects of alcohol, exhibit significant differences in sensitivity to nicotine [33]. Other rodent lines selected for differences in various alcohol-related behaviors also have been found to differ in sensitivity to nicotine [10,11,13,16,17,23].

One potential site of convergence of the actions of nicotine and alcohol is the family of neuronal nicotinic acetylcholine receptors (nAChRs) expressed in the brain. In mammals, the neuronal subfamily of nAChRs comprise an indeterminate number of subtypes that are composed of pentameric combinations of the subunits α2–α7, α9, α10, and β2–β4 [25]. Söderpalm et al. [35] demonstrated in rats that activation of nAChRs in the ventral tegmental area of the brain is involved in the dopamine-activating and rewarding effects of alcohol. Others have established that various nAChR subtypes are functionally modulated by alcohol [1,6,46]. The subtypes of nAChRs whose function is modulated by alcohol include both α4β2 [1,6,46] and α7 [6,44] nAChRs, the two most abundant nAChR subtypes found in the brain. Individual differences in the expression of these two receptor subtypes have been correlated with various responses to nicotine [27,31] and alcohol [39] in rodents. Moreover, a polymorphism in the mouse nAChR α4 subunit gene, *Chrna4* [37], has been shown to be associated with various behavioral responses to nicotine [36,41,42] and alcohol [41]. The *Chrna4* polymorphism also influences the function of α4β2 nAChRs in mouse brain synaptosomes [14] and affects α4β2 nAChR pharmacology when evaluated in a heterologous system [24]. Recently, Butt et al. [5] demonstrated that the *Chrna4* polymorphism is associated with the ability of alcohol to potentiate the function of α4β2 nAChRs in mouse brain synaptosomes. These data suggest that genetically-determined differences in the expression and/or function of these nAChR subtypes might be responsible for at least some of the genetic overlap between nicotine and alcohol-related behaviors.

A few reports have described both nicotine and alcohol consumption in rodents. These studies each found a significant relationship between nicotine and alcohol consumption. However, drug consumption in these studies was only compared between either two inbred mouse strains [29], two selected rat lines [40] or transgenic mice that over express bovine growth hormone and their control littermates [30]. In order to more fully assess the genetic overlap between nicotine and alcohol consumption in mice, free-choice nicotine and alcohol consumption was measured using a two-bottle ascending drug concentration paradigm in 50 F2 intercross mice derived from the inbred strains C57BL/6J and C3H/HeJ. These two parental strains are known to differ in both nicotine and alcohol consumption [4,32]. The potential role of nAChRs in influencing both nicotine and alcohol consumption was also evaluated. For this, levels of α4β2 and α7 nAChRs were measured in all mice by radio-ligand binding assays in four brain regions (cortex, hippocampus, midbrain and striatum). In addition, the relationship between *Chrna4* genotype and the consumption of nicotine and alcohol was evaluated in these animals.

2. Methods

2.1. Animals

C57BL/6J, C3H/HeJ, and (C57BL/6J female × C3H/HeJ male) F1 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6J × C3H/HeJ F2 intercross mice were produced in-house by mating the F1 animals purchased from The Jackson Laboratory. The F2 animals were weaned at 21 days and group housed by sex. The mice were maintained on a 12 h light/dark schedule and had free access to food (Harlan Teklad, Indianapolis, IN) and water. For preference testing, mice were singly housed in a standard mouse cage and provided with food and two bottles of fluid. One bottle contained the test drug dissolved in water and the other bottle contained water only. Each drug concentration was tested for a period of 4 days and the bottle positions were rotated every day. The mass of each animal was measured at the beginning and end of each drug concentration. For all animals, alcohol preference was measured first starting with 3% alcohol, followed immediately by 6% alcohol and then 10% alcohol. Six days following the completion of the 10% alcohol trial, nicotine preference was initiated. Between the alcohol and nicotine trials, the mice only had access to water. The first nicotine concentration tested was 25 μg/ml nicotine solution followed immediately by 50 and 100 μg/ml nicotine solutions.

2.2. Receptor binding

The binding of [^{3}H]-cytisine to particulate fractions from cortex, hippocampus, midbrain and striatum was measured using methods similar to those described for [^{3}H]-nicotine binding in Marks et al. [28]. Particulate fractions obtained from P2 preparations of the four brain regions were incubated with 10 nM [^{3}H]-cytisine in 100 μl of Krebs–Ringers–HEPES (118 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 20 mM HEPES, pH 7.4) (KRH) for 1.5 h at 4 °C. Incubations were conducted in 96-well polystyrene plates. Non-specific binding was determined by including 10 μM unlabeled (−)-nicotine in the incubation. The binding reaction was terminated by filtration of the particulate fractions onto glass fiber filters that were soaked in 0.5% polyethylenimine in KRH. After filtration, the filters were washed six times with ice-cold KRH. All filtration was done using a Tomtec (Hamden, CT) Mach II harvester. The filters were collected and placed in scintillation vials. Following the addition of scintillation fluid, the radioactivity was measured using a liquid scintillation counter. The binding of [^{125}I]-α-bungarotoxin ([^{125}I]-αBTX) was performed as described for [^{3}H]-cytisine binding with the following exceptions. Particulate fractions were incubated with 2 nM [^{125}I]-αBTX for 3 h at 37 °C and non-specific binding was determined by the inclusion of 1 mM (−)-nicotine in the incubation. Filtration of the samples was done using glass fiber filters that were soaked in 0.5% polyethylenimine and 5%

non-fat dry milk in KRH. Filters were counted on a Packard Cobra II gamma counter (Perkin-Elmer, Boston, MA). Homogenate protein levels were determined by the method of Lowry et al. [26].

2.3. Chrna4 genotyping

Genomic DNA from the 50 F2 mice was isolated from splenic tissue by standard proteinase K digestion/phenol extraction methodology as described previously [36]. A region of *Chrna4* that spanned the SNP at nucleotide position 1587 was amplified by a reaction that included 50 ng of genomic DNA, 1× PCR buffer II (PE Biosystems, Foster City, CA), 2.5 mM $MgCl_2$, 200 μM each, dGTP, dATP, dCTP, dTTP, 20 pmol of each amplification primer (5′-GGTCCCTGAGCGTCCAGCATG-3′ and 5′-GGTCCTATCTGGGTCGGGGTG-3′), 2.5 units Amplitaq Gold DNA polymerase (PE Biosystems) in a reaction volume of 50 μl. Amplification of the DNA was accomplished using a touchdown protocol with an initial annealing temperature of 65 °C and final amplification conditions of 94 °C, 30 s; 55 °C, 30 s; 72 °C, 1 min, for 30 cycles. This amplification reaction generates a product of 405 bp that spans from 185 bp upstream of the *Chrna4* SNP at nucleotide position 1587 to 220 downstream of this SNP. Following amplification, 5 μl of the PCR reaction was digested with *Stu*I in a final volume of 20 μl and subsequently electrophoresed on a 1.8% agarose gel. The restriction enzyme *Stu*I (recognition sequence AG**GCC**T) will cut the PCR product if the alanine codon, GCC, is present at codon position 529 but will not cut the PCR product if the threonine codon, ACC, is present at this position.

2.4. Statistics

All statistical analyses were performed using the SPSS 12.0 software package (SPSS, Chicago, IL). Consumption measures (drug consumption and preference ratio) for both nicotine and alcohol in the inbred strains were assessed using a general linear model for repeated measures. Consumption measures for both nicotine and alcohol in the F2 intercross mice were evaluated using two-way ANOVA. The relationship between nicotine and alcohol consumption as well as the relationship between drug consumption and [^{3}H]-cytisine and [^{125}I]-αBTX binding levels were evaluated using bivariate correlational analysis (Pearson's two-tailed correlation coefficient). The relationship between *Chrna4* genotype and drug intake was performed using the general linear model for repeated measures.

3. Results

3.1. Alcohol consumption in C57BL/6J and C3H/HeJ inbred mice

Alcohol consumption was measured in C57BL/6J and C3H/HeJ mice using the two-bottle choice paradigm as described in Section 2. Testing was conducted over a 12-day period. During each of three separate 4-day periods, the mice were provided with bottles containing water and water supplemented with 3% (v/v) alcohol (first 4 days), 6% (v/v) alcohol (second 4 days) and 10% (v/v) alcohol (last 4 days). For the alcohol preference measure (Fig. 1A), significant between-subject effects were observed for strain

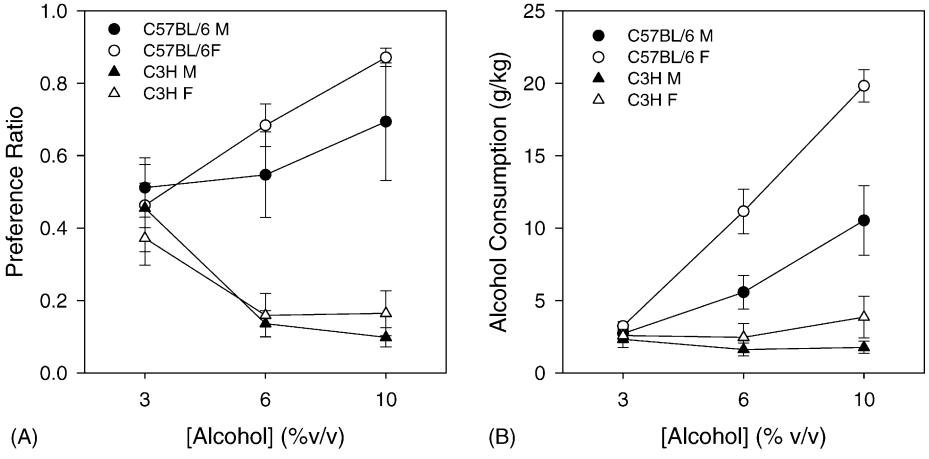

Fig. 1. Measurement of alcohol preference and alcohol consumption in male and female C57BL/6 and C3H/HeJ mice. Male and female mice of the inbred mouse strains C57BL/6J (n = 5 males and 5 females) and C3H/HeJ (n = 6 males and 6 females) were tested in an ascending alcohol two-bottle choice paradigm and preference ratios (percent of total fluid consumed from the alcohol-containing bottle) and alcohol consumption (grams alcohol consumed/kg body mass) were determined for each alcohol concentration. (A) Alcohol preference. For alcohol preference ratio, a significant between-subject effects were observed for strain ($P < 0.001$), but not for sex. A significant concentration-strain interaction was detected for within-subject effects ($P < 0.001$). (B) Alcohol consumption. Between-subject measures for alcohol consumption detected significant effects of strain ($P < 0.001$), sex ($P < 0.001$) and a strain–sex interaction ($P < 0.05$). Significant effects of alcohol concentration ($P < 0.001$), and concentration × strain ($P < 0.001$), concentration × sex ($P < 0.005$) and concentration × strain × sex ($P < 0.05$) interactions were observed for within-subject tests. All data are presented as mean ± S.E.M.

($F_{(1,22)}$ = 48.47, $P < 0.001$), but not for sex. A significant concentration-strain interaction was detected for within-subject effects ($F_{(2,22)}$ = 16.76, $P < 0.001$). Between-subject measures for alcohol consumption (Fig. 1B) detected significant effects of strain ($F_{(1,22)}$ = 71.26, $P < 0.001$), sex ($F_{(1,22)}$ = 16.64, $P < 0.001$) and a strain–sex interaction ($F_{(1,22)}$ = 7.19, $P < 0.05$). Significant effects of alcohol concentration ($F_{(2,22)}$ = 47.2, $P < 0.001$), and concentration × strain ($F_{(2,22)}$ = 41.4, $P < 0.001$), concentration × sex ($F_{(2,22)}$ = 8.38, $P < 0.005$) and concentration × strain × sex ($F_{(2,22)}$ = 3.66, $P < 0.05$) interactions were observed for within-subject tests.

3.2. Nicotine consumption in C57BL/6J and C3H/HeJ inbred mice

Nicotine consumption also was measured in C57BL/6J and C3H/HeJ mice using the two-bottle choice paradigm as described in Section 2. Testing was conducted over a 12-day period. During each of three separate 4-day periods, the mice were provided with bottles containing water and water supplemented with 25 μg/ml nicotine (first 4 days), 50 μg/ml nicotine (second 4 days) and 100 μg/ml nicotine (last 4 days). A significant between-subject effect was observed for nicotine preference for strain ($F_{(1,20)}$ = 10.48, $P < 0.005$), but not for sex (Fig. 2A). A significant effect of concentration also was detected for within-subject effects ($F_{(2,20)}$ = 33.62, $P < 0.001$). Between-subject measures for nicotine consumption (Fig. 2B) detected significant effects of strain ($F_{(1,20)}$ = 17.52, $P < 0.001$) and a strain–sex interaction ($F_{(1,20)}$ = 7.81, $P < 0.01$). Significant effects of nicotine concentration ($F_{(2,20)}$ = 3.78, $P < 0.05$), and concentration × strain ($F_{(2,20)}$ = 14.75, $P < 0.001$), and concentration × strain × sex ($F_{(2,20)}$ = 3.8, $P < 0.05$) interactions were observed for within-subject tests of nicotine consumption.

3.3. Effect of sequential alcohol and nicotine two-bottle choice tests on measures of nicotine preference

In order to evaluate the relationship between the consumption of alcohol and nicotine in the same animal, sequential testing of the two drugs is necessary. Therefore, C57BL/6J and C3H/HeJ mice were evaluated for whether prior testing of mice with the alcohol two-bottle choice paradigm affects measures of nicotine preference in a subsequent nicotine two-bottle choice test. Female and male mice of both inbred strains were tested in the 12-day, two-bottle preference paradigm for alcohol exactly as described earlier. An equal number of animals were tested simultaneously in the two-bottle paradigm in which both bottles contained water only. Six days following the completion of the water/alcohol, water/water two-bottle choice experiments, the mice were tested for nicotine two-bottle choice exactly as described earlier. Although alcohol exposure preceding nicotine preference showed a tendency to decrease nicotine consumption in C57BL/6 mice and to a lesser extent in C3H/HeJ mice, statistical analysis of nicotine preference (Fig. 3A) and consumption (Fig. 3B) between mice that were or were not exposed to alcohol in the initial two-bottle choice test indicated that exposure to alcohol did not significantly alter either nicotine preference ($F_{(1,42)}$ = 0.174, $P > 0.5$) or nicotine consumption ($F_{(1,42)}$ = 1.12, $P > 0.2$) in either strain.

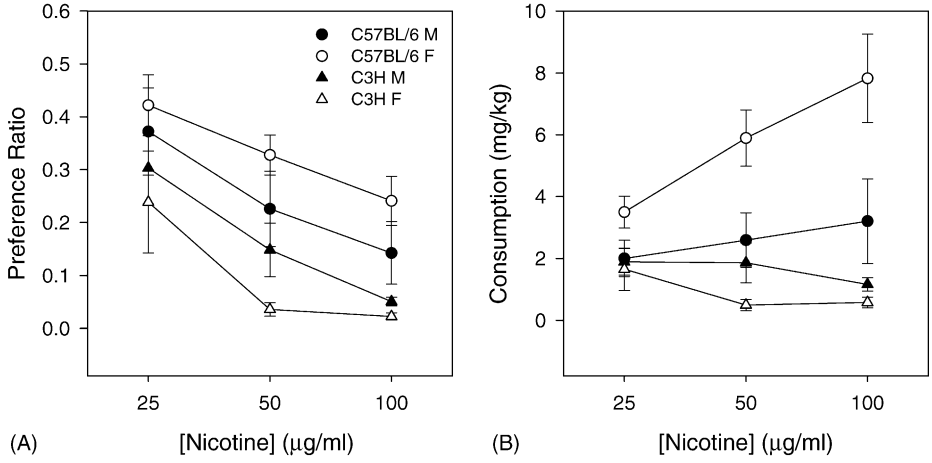

Fig. 2. Measurement of nicotine preference and nicotine consumption in male and female C57BL/6 and C3H/HeJ mice. Male and female mice of the inbred mouse strains C57BL/6J (n = 5 males and 5 females) and C3H/HeJ (n = 6 males and 4 females) were tested in an ascending nicotine two-bottle choice paradigm and preference ratios (percent of total fluid consumed from the nicotine-containing bottle) and nicotine consumption (mg nicotine consumed/kg body mass) were determined for each nicotine concentration. (A) Nicotine preference. A significant between-subject effect was observed for nicotine preference for strain ($P < 0.005$), but not for sex. A significant effect of concentration also was detected for within-subject effects ($P < 0.001$). (B) Nicotine consumption. Between-subject measures for nicotine consumption detected significant effects of strain ($P < 0.001$) and a strain–sex interaction ($P < 0.01$). Significant effects of nicotine concentration ($P < 0.05$), and concentration × strain ($P < 0.001$), and concentration × strain × sex ($P < 0.05$) interactions were observed for within-subject tests of nicotine consumption. All data are presented as mean ± S.E.M.

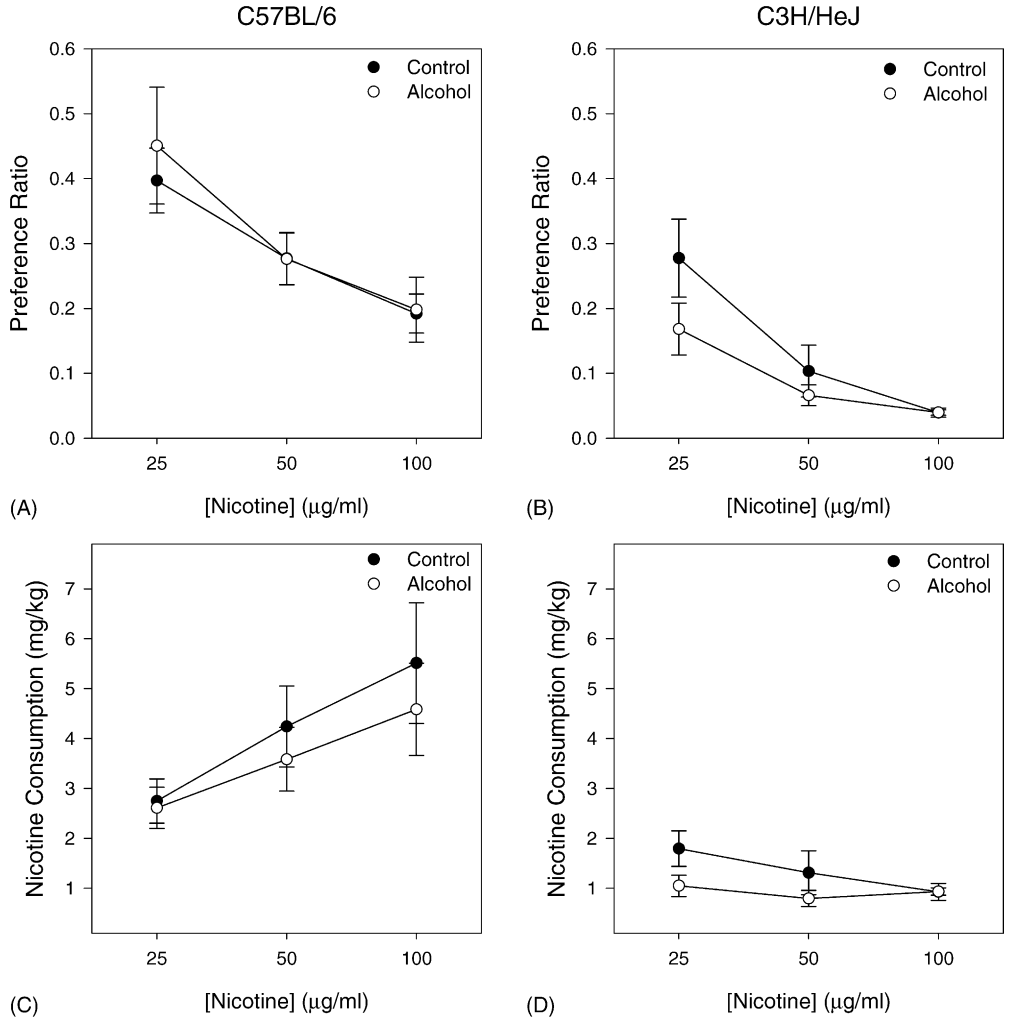

Fig. 3. Effect of prior exposure to alcohol on nicotine preference and consumption in C57BL/6J and C3H/HeJ mice. Prior to performing the ascending nicotine two-bottle choice test, C57BL/6J and C3H/HeJ mice were exposed to the ascending alcohol two-bottle choice test (n = 5 males and 5 females for C57BL/6J and 6 males and 6 females for C3H/HeJ) or the two-bottle choice test in which both bottles contained only water (n = 5 males and 5 females for C57BL/6J and 6 males and 4 females for C3H/HeJ). A comparison of nicotine preference (panels A and B) and consumption (panels C and D) between mice that were or were not exposed to alcohol in the initial two-bottle choice test indicated that exposure to alcohol did not alter either nicotine preference ($P > 0.5$) or nicotine consumption ($P > 0.2$). All data are presented as mean ± S.E.M.

3.4. Alcohol consumption in F2 intercross mice

Alcohol consumption was measured in 50 F2 intercross animals (26 female and 24 male mice) using the two-bottle choice paradigm as described in Section 2 (Fig. 5). Testing was conducted over a 12-day period. During each of three separate 4-day periods, the mice were provided with bottles containing water and water supplemented with 3, 6, and 10% (v/v). Female mice consumed 49.8 ± 3.8, 57.3 ± 5.3, and 61.3 ± 6.1% of their total fluid from the 3, 6, and 10% ethanol solutions, respectively (Fig. 4). In contrast, male mice consumed 28.2 ± 4.1, 26.3 ± 5.5, and 22.9 ± 5.7% of their total fluid from the alcohol-containing solutions. The average consumption of alcohol during the 3, 6, and 10% trials was 6.16 ± 0.62, 7.23 ± 0.8, and 12.0 ± 1.2 g/kg for female mice and 2.53 ± 0.37, 2.39 ± 0.49, and 3.31± 0.81 g/kg for male mice. A significant effect of sex was detected for both the alcohol preference ratio measure ($P < 0.001$) and consumption of alcohol ($F_{(1,50)} = 60.31$, $P < 0.001$). There was also a significant effect of alcohol concentration on the consumption of alcohol ($P < 0.001$) and a significant sex–alcohol concentration interaction ($P < 0.001$).

3.5. Nicotine consumption in F2 intercross mice

After completion of the two-bottle choice test with alcohol, nicotine consumption was measured in the same 50 C57BL/6 × C3H/2 F2 intercross mice using the two-bottle choice paradigm as described in Section 2. During the test period, the mice were provided with bottles containing water and water supplemented with 25 μg/ml nicotine for the first 4 days, water and water supplemented with 50 μg/ml

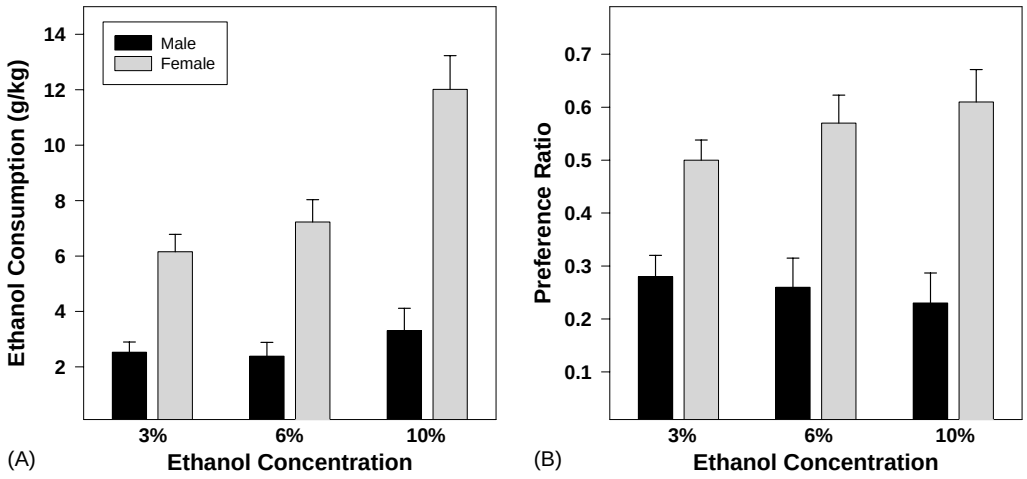

Fig. 4. Relationships between ethanol concentrations and ethanol consumption or preference ratio in male and female C57BL/6 × C3H/HeJ F2 intercross mice. Fifty F2 intercross mice (26 female, 24 male) were evaluated for free-choice alcohol consumption in the two-bottle choice paradigm exactly as performed for the parent strains. (A) Female mice consumed more alcohol at all three alcohol concentrations tested relative to their male counterparts ($P < 0.001$). A significant effect of alcohol concentration on the consumption of alcohol was also detected ($P < 0.001$). (B) There was a significant sex effect on the ethanol preference ratio ($P < 0.001$). Female mice exhibited a greater preference for alcohol than did male mice. However, the preference ratio did not differ for either male or female mice at the three concentrations of alcohol tested. All data are presented as mean ± S.E.M.

nicotine for the next 4 days and water and water supplemented with 100 μg/ml for the last 4 days. During the 25, 50, and 100 μg/ml trials, female mice consumed 43.7 ± 2.4, 29.9 ± 3.8, and 14.3 ± 2.5% of their total fluid from the nicotine-laced solution, respectively (Fig. 5). Male mice consumed 38.4 ± 2.9, 28.0 ± 3.8, and 13.6 ± 2.4%, respectively, of their total fluid from the nicotine-containing solutions. The average consumption of nicotine during the 25, 50, and 100 μg/ml trials was 2.74 ± 0.17, 3.79 ± 0.45, and 3.62 ± 0.58 mg/kg for female mice and 2.02 ± 0.18, 2.96 ± 0.39, and 2.7 ± 0.5 mg/kg for male mice. There was a significant effect of nicotine concentration on the preference ratio ($P < 0.001$) but not for nicotine consumption. Although female mice tended to consume more nicotine at each nicotine concentration relative to their male counterparts, there was no significant effect of sex on nicotine consumption.

3.6. Relationship between alcohol and nicotine consumption

In order to evaluate whether individual differences in alcohol consumption were related to individual differences in

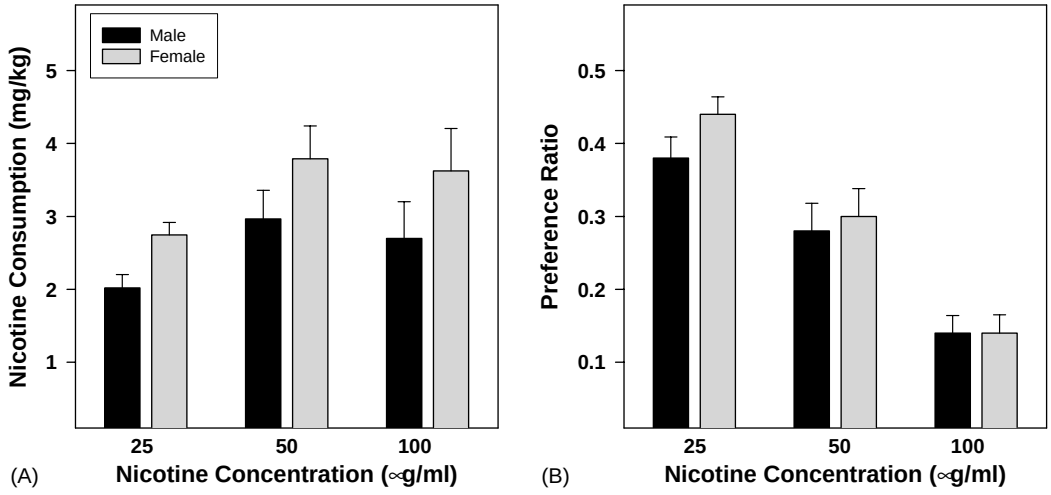

Fig. 5. Relationships between nicotine concentrations and dose of nicotine consumed or preference ratio in male and female C57BL/6 × C3H/HeJ F2 intercross mice. The same 50 F2 intercross mice tested for alcohol preference were tested for nicotine preference in the two-bottle choice paradigm. (A) Female mice tended to consume more nicotine than their male counterparts during the 25, 50, and 100 μg/ml trials, but the differences were not significant ($P = 0.094$). The consumption of nicotine did not differ significantly between the three concentrations of nicotine tested. (B) No sex differences were detected for the nicotine preference ratio while the concentration of nicotine tested did have a significant effect on the preference ratio ($P < 0.001$). All data are presented as mean ± S.E.M.

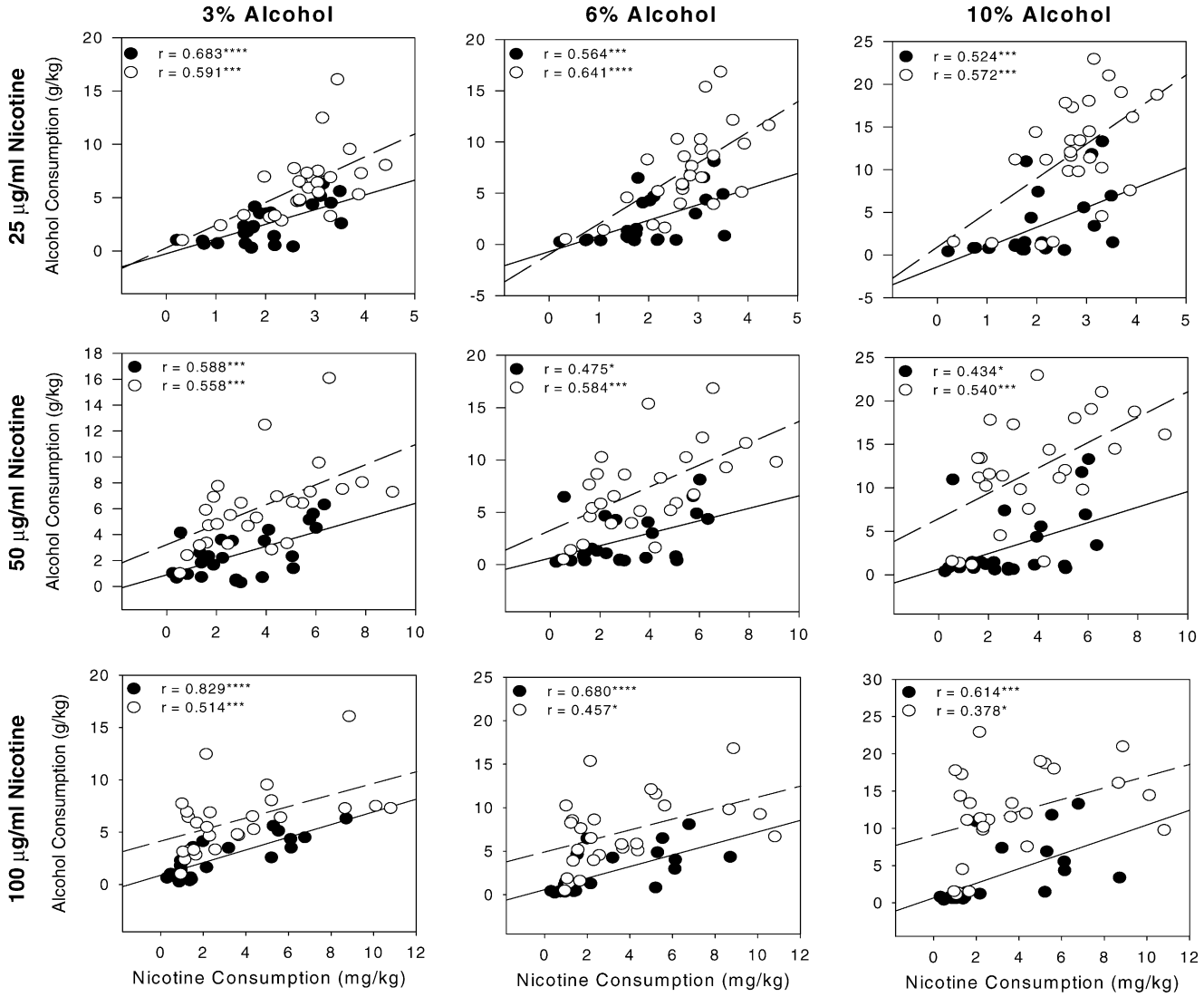

Fig. 6. Correlation between nicotine and alcohol consumption in C57BL/6 × C3H/HeJ F2 intercross mice. The relationship between individual alcohol and nicotine consumption was evaluated in the F2 intercross mice. Correlations for both male (●) and female (○) mice are shown. The consumption of alcohol and nicotine was significantly correlated in male and female mice for all pair wise comparisons of the tested alcohol and nicotine concentrations. The mean correlation (average of all pair wise combinations) between alcohol and nicotine consumption was 0.599 for male mice and 0.537 for female mice.

nicotine consumption, the two measures were compared at all three concentrations of each drug (Fig. 6). Since sex differences were detected for the alcohol consumption measures, bivariate correlational analysis was performed separately for the female and male mice. For all concentrations of nicotine and alcohol tested, nicotine consumption was significantly correlated with alcohol consumption in both female and male mice. The highest correlation between alcohol and nicotine consumption in female mice was observed for the comparison between 25 μg/ml nicotine and 6% alcohol (0.641, $P < 0.001$) and the lowest correlation for these measures in female mice was between 100 μg/ml nicotine and 10% alcohol (0.378, $P < 0.05$). In male mice, the highest correlation between alcohol and nicotine consumption was observed for the comparison between 100 μg/ml nicotine and 3% alcohol (0.829, $P < 0.001$) and the lowest correlation for these measures in male mice was between 50 μg/ml nicotine and 10% alcohol (0.434, $P < 0.05$). These results suggest that in females, between 41% (25 μg/ml nicotine versus 6% alcohol) and 14% (100 μl/ml versus 10% alcohol) of the variance in nicotine and alcohol consumption may be attributed to common genetic factors. In comparison, between 69% (100 μg/ml nicotine versus 3% alcohol) and 19% (50 μg/ml nicotine versus 10% alcohol) of the variance in nicotine and alcohol consumption in male mice may be attributed to common genetic factors. Similar results were obtained for comparisons between alcohol and nicotine preference ratios (data not shown).

3.7. Nicotinic receptor levels and drug consumption

In order to assess whether individual differences in nicotinic receptor levels are correlated with drug consumption,

Table 1
Relationship between nicotinic receptor levels and measures of nicotine or alcohol consumption

	Cortex		Hippocampus		Striatum		Midbrain	
	Cytisine	αBTX	Cytisine	αBTX	Cytisine	αBTX	Cytisine	αBTX
E3% ratio	0.025/0.075	0.146/0.225	−0.137/−0.103	−0.195/−0.226	−0.100/−0.138	−0.168/−0.027	0.027/0.238	−0.001/−0.261
E6% ratio	−0.32/0.080	0.140/0.286	−0.085/−0.062	−0.178/−0.198	0.013/−0.033	−0.141/0.085	0.069/0.263	−0.073/−0.159
E10% ratio	−0.026/0.043	−0.054/0.150	−0.041/−0.180	−0.263/−0.033	0.121/−0.043	0.010/−0.084	0.067/0.250	−0.061/−0.261
E3% con	0.034/0.055	0.147/0.294	−0.103/−0.123	−0.161/−0.171	−0.144/−0.074	−0.225/0.040	0.010/0.264	0.081/−0.279
E6% con	0.009/0.111	0.058/0.336	−0.058/−0.157	−0.173/−0.148	−0.074/−0.050	−0.236/0.078	0.031/0.243	0.026/−0.181
E10% con	−0.009/0.086	−0.008/0.192	−0.044/−0.253	−0.205/−0.005	0.039/−0.026	−0.122/−0.042	0.025/0.255	−0.024/−0.253
N25 ratio	0.064	0.109	−0.254	−0.187	0.213	0.003	0.111	−0.051
N50 ratio	0.261	0.287	−0.214	−0.287	0.066	0.029	0.168	0.057
N100 ratio	0.183	0.342*	−0.299*	−0.163	−0.027	0.078	0.065	0.087
N25 con	0.107	0.081	−0.258	−0.175	0.079	−0.196	0.109	−0.172
N50 con	0.193	0.183	−0.289*	−0.203	−0.014	−0.124	0.095	−0.067
N100 con	0.185	0.307*	−0.328*	−0.125	−0.05	−0.025	0.091	0.027

Cytisine: [^{3}H]-cytisine binding; αBTX: [^{125}I]-α-bungarotoxin binding; E3%: 3% ethanol; E6%: 6% ethanol; E10%: 10% ethanol. For ethanol, correlations with both female (given first) and male data are shown. N25: 25 μg/ml nicotine; N50: 50 μg/ml nicotine; N100: 100 μg/ml nicotine; ratio: preference ratio; con: average daily consumption (g/kg alcohol, mg/kg nicotine).
* $P < 0.05$.

the binding of [^{3}H]-cytisine and [^{125}I]-αBTX were measured in cortex, striatum, midbrain and hippocampus in the 50 F2 mice (Table 1). Due to sex differences in the alcohol measures, correlations between receptor levels and the alcohol phenotypes were made for each sex. No significant correlations were detected between levels of either [^{3}H]-cytisine or [^{125}I]-αBTX and alcohol preference or consumption. Relatively low but significant correlations were observed between the measures of nicotine consumption at the highest nicotine concentration tested and the level of [^{3}H]-cytisine binding sites in the hippocampus and [^{125}I]-αBTX binding sites in the cortex.

3.8. Nicotinic receptor genotype and drug consumption

In order to determine the relationship between a previously identified polymorphism in the nAChR subunit gene *Chrna4* [37] and drug consumption, the *Chrna4* genotype of each animal was determined and compared to drug consumption. Although there was a trend towards lower nicotine preference and consumption in mice homozygous for the C3H allele of *Chrna4*, no significant association between *Chrna4* genotype and either nicotine preference ratio or dose of nicotine consumed was observed (Fig. 7). In contrast, *Chrna4* genotype was significantly associated with the alcohol consumption ratio ($P < 0.01$) and dose of alcohol consumed (P

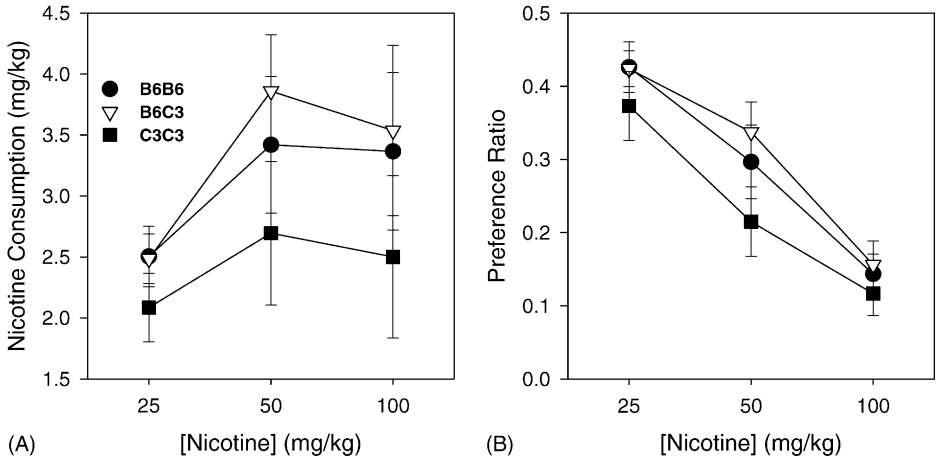

Fig. 7. Association between *Chrna4* genotype and nicotine consumption in C57BL/6 × C3HHeJ F2 intercross mice. The 50 F2 mice were genotyped for a previously identified polymorphism in the nicotinic receptor subunit gene, *Chrna4* as described Section 2 and the relationship between *Chrna4* genotype and measures of nicotine intake were evaluated. Although mice homozygous for the C3H allele of *Chrna4* tended to consume less nicotine than heterozygous mice or C57BL/6 allele homozygotes, the relationship between the *Chrna4* polymorphism and either nicotine consumption (A) or the nicotine preference ratio (B) was not significant. B6B6, mice homozygous for the C57BL/6J allele of *Chrna4*; B6C3, mice heterozygous for the *Chrna4* alleles; C3C3, mice homozygous for the C3H/HeJ allele of *Chrna4*. All data are presented as mean ± S.E.M.

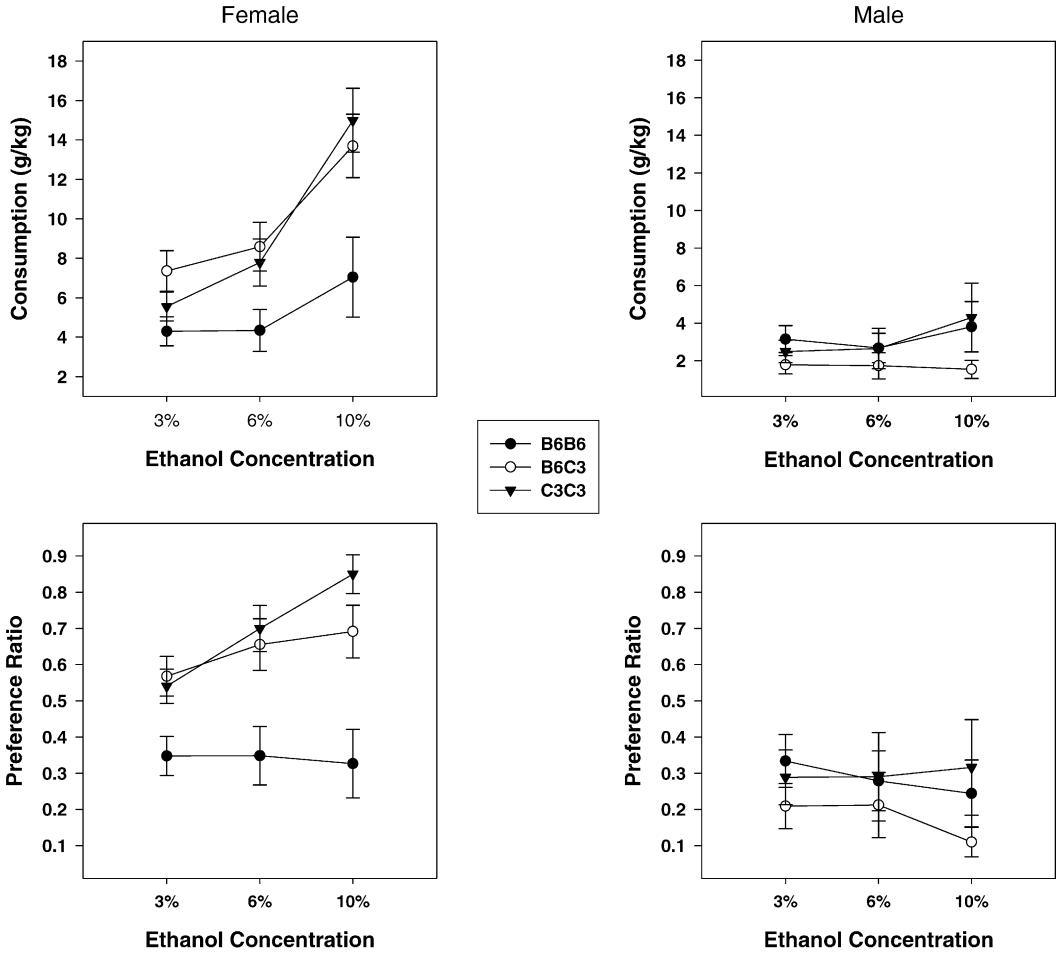

Fig. 8. Association between the *Chrna4* genotype and ethanol consumption in C57BL/6 × C3H2/HeJ F2 intercross mice. The 50 F2 mice were genotyped for the polymorphism in *Chrna4* and the relationship between *Chrna4* genotype and measures of alcohol intake were evaluated. *Chrna4* genotype was significantly associated with alcohol consumption ($P < 0.001$) and the alcohol preference ratio ($P < 0.001$) in female, but not male mice. B6B6, mice homozygous for the C57BL/6J allele of *Chrna4*; B6C3, mice heterozygous for the *Chrna4* alleles; C3C3, mice homozygous for the C3H/HeJ allele of *Chrna4*. All data are presented as mean ± S.E.M.

< 0.05) in females (Fig. 8). Female mice homozygous for the C57BL/6 allele of *Chrna4* exhibited less preference for alcohol and consumed less alcohol relative to mice heterozygous for *Chrna4* or homozygous for the C3H allele of *Chrna4*. A significant relationship between *Chrna4* genotype and the alcohol consumption measures was not observed in male mice.

4. Discussion

The results of this study provide evidence that there are common genetic determinants for the consumption of nicotine and alcohol in mice. This is the first demonstration of a correlation between alcohol and nicotine consumption in a genetically segregating population of mice. Significant genetic correlations were observed between nicotine and alcohol consumption at all concentrations of nicotine and alcohol examined. In female mice, the correlations ranged from 0.641 ($P < 0.001$) for 25 μg/ml nicotine versus 6% alcohol to 0.378 ($P < 0.05$) for 100 μg/ml nicotine versus 10% alcohol. The correlations between the alcohol and nicotine measures in male mice ranged from 0.829 ($P < 0.001$) for 100 μg/ml nicotine versus 3% alcohol to 0.434 ($P < 0.05$) for 50 μg/ml nicotine and 10% alcohol. The average correlation between alcohol and nicotine consumption was 0.537 for females and 0.599 for males. Therefore, taking all alcohol–nicotine comparisons into account, approximately 29 and 36% of the phenotypic variance in alcohol and nicotine consumption may be attributed to common genetic factors in female and male mice, respectively.

Another potential explanation for the significant correlation between alcohol and nicotine consumption is that prior exposure to alcohol "primes" the mice that drink the alcohol to drink nicotine. However, this does not appear to be the case as previous exposure to alcohol did not significantly effect subsequent nicotine consumption in either of the inbred strains used to generate the F2 mice used in this study. In fact, exposure to alcohol prior to nicotine may actually

slightly decrease nicotine consumption (although the effect was not significant). This effect of prior alcohol exposure on nicotine consumption might actually lead to an artificially low correlation between the measures of alcohol and nicotine consumption.

Levels of the nicotinic receptor subtypes α4β2 and α7, as measured by cytisine and αBTX binding, respectively, were found to correlate with a few measures of nicotine consumption but not with any measures of alcohol consumption. The lack of a significant correlation between striatal cytisine and αBTX binding with alcohol consumption is in contrast to the results of Tizabi et al. [39]. This group demonstrated that the alcohol-preferring selected rat line exhibits significantly lower levels of both cytisine and αBTX binding sites in striatum as compared to their non-preferring selected rat line counterparts. The combination of these data indicates that the relationship between levels of nicotinic receptors and alcohol and nicotine consumption are dependent upon the test population. The low correlation values observed between receptor levels and nicotine consumption indicate that individual differences in nicotinic receptor levels contribute to only a minor fraction of the phenotypic variance. Furthermore, individual differences in receptor levels cannot explain the genetic correlation between alcohol and nicotine consumption in the population used in this study.

Polymorphisms in the nicotinic receptor subunit gene *Chrna4* also cannot account for the genetic correlation between alcohol and nicotine consumption in the C57BL/6J × C3H/HeJ F2 mice. Although a trend was observed for the relationship between *Chrna4* genotype and nicotine consumption, the trend was not significant. Recently, Butt et al. (submitted for publication) demonstrated that *Chrna4* genotype was significantly associated with nicotine consumption in an F2 intercross between C57BL/6J and A/J mice. The A/J mouse strain possesses the same *Chrna4* allele as C3H/HeJ [24]. Like the data reported in this study, F2 mice homozygous for the C57BL/6J allele of *Chrna4* consumed the most nicotine. Butt et al. also found that the association between *Chrna4* genotype and nicotine consumption was eliminated in F2 mice that carried a null mutation for the gene, *Chrnb2*, which encodes the β2-nicotinic receptor subunit. Most, if not all, α4-containing nicotinic receptors co-assemble with the β2 subunit in the brain. Therefore, the finding that the association between *Chrna4* genotype and nicotine preference is not observed when nicotinic receptors that contain the α4 subunit are absent from the brain provides evidence that the association between nicotine preference and *Chrna4* genotype is due to the polymorphism in *Chrna4* and not due to a gene linked to *Chrna4*.

The lack of a significant association between *Chrna4* genotype and nicotine consumption in the C57BL/6J × C3H/HeJ F2 mice could indicate that the polymorphism in *Chrna4* does not influence nicotine consumption in this particular population. Alternatively, the lack of significance, despite the same trend in the relationship between *Chrna4* genotype and nicotine consumption, could be due to a lack of statistical power in the C57BL/6J × C3H/HeJ F2 population. Reduced power to detect an association between *Chrna4* genotype and nicotine consumption might be explained by the observation that C57BL/6J and C3H/HeJ F2 mice also are polymorphic for the genes that encode the α6 nicotinic receptor subunit (*Chrna6*) and the β3 nicotinic receptor subunit (*Chrnb3*) (J. Stitzel, unpublished data). C57BL/6J and A/J F2 mice do not differ at the *Chrna6* and *Chrnb3* loci. One of the predominant nicotinic receptor subtypes expressed in the so-called reward pathway is comprised of the subunits α4, α6, β2, and β3 [45,8,34]. If this receptor subtype is important for nicotine consumption, then polymorphisms in any of these subunits might affect consumption. Consequently, in the C57BL/6J × A/J F2 mice, function of this receptor subtype would be affected only by the strain variants of the α4 subunit. In contrast, the influence of the variants of the α4 subunit on the function of the putative α4α6β2β3 nicotinic receptor in C57BL/6J × C3H/HeJ F2 mice might be affected by which strain-specific variants of the α6 and β3 subunits are included in the receptor.

Chrna4 genotype was associated with alcohol consumption although the association was observed in female mice only. Female mice homozygous for the C3H allele of *Chrna4* consumed more alcohol than female mice homozygous for the C57BL/6 allele of *Chrna4*. A potential role for *Chrna4* in alcohol preference previously has been indicated. Gill et al. [15] identified a QTL for preference for 10% alcohol in the A/J × C57BL/6 (AXB) and C57BL/6 × A/J (BXA) recombinant inbred strains that mapped to distal chromosome 2, near the *Chrna4* locus. The mouse strain A/J carries the same allele for *Chrna4* as C3H/HeJ. In contrast to the results of the current study, the association between distal chromosome 2/*Chrna4* and alcohol consumption was observed in both males and females. Moreover, the direction of the effect of distal chromosome 2/*Chrna4* was in the opposite direction relative to the C57BL/6 genotype. In the study by Gill et al. [15], the C57BL/6 allele was associated with increased alcohol consumption while the C57BL/6 allele in the C57BL/6 × C3H F2 mice was associated with reduced alcohol consumption. Butt et al. (submitted for publication) also demonstrated that *Chrna4* is associated with alcohol consumption in a C57BL/6 × A/J F2 intercross. Like the results of Gill et al. [15], the study by Butt et al. (submitted for publication) found that the C57BL/6 allele of *Chrna4* was associated with higher alcohol preference. However, Butt et al. found that the association between *Chrna4* genotype and alcohol preference was maintained in mice that did not express the nicotinic receptor β2 subunit. Therefore, the association between alcohol preference and *Chrna4* is likely due to a gene that is linked to *Chrna4*. This may explain why the direction of the effect of distal chromosome 2/*Chrna4* and the influence of sex on the relationship between *Chrna4* genotype and alcohol consumption appears to be dependent upon the genetic background of the test population. The A/J and C3H/HeJ strains may possess different alleles of a gene linked to *Chrna4* that have opposite effects on alcohol consumption.

In summary, the results presented here indicate that there are common genetic influences on nicotine and alcohol consumption in mice. However, the genetic overlap between nicotine and alcohol consumption in mice cannot be attributed to individual differences in expression of α4β2 or α7 nAChRs or to a polymorphism in the nAChR subunit gene *Chrna4*. Consequently, the genetic basis for the relationship between nicotine and alcohol consumption remains to be determined.

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References

[1] Aistrup GL, Marszalec W, Narahashi T. Ethanol modulation of nicotinic acetylcholine receptor currents in cultured cortical neurons. Mol Pharmacol 1999;55:39–49.
[2] Batel P, Pessione F, Maitre C, Rueff B. Relationship between alcohol and tobacco dependencies among alcoholics who smoke. Addiction 1995;90:977–80.
[3] Battjes RJ. Smoking as an issue in alcohol and drug-abuse treatment. Addict Behav 1988;13:225–30.
[4] Belknap JK, Crabbe JC, Young ER. Voluntary consumption of ethanol in 15 inbred mouse strains. Psychopharmacology 1993;112:503–10.
[5] Butt CM, Hutton SR, Stitzel JA, Balogh SA, Owens JC, Collins AC. A polymorphism in the alpha4 nicotinic receptor gene (*Chrna4*) modulates enhancement of nicotinic receptor function by ethanol. Alcohol Clin Exp Res 2003;27:733–42.
[6] Cardoso RA, Brozowski SJ, Chavez-Noriega LE, Harpold M, Valenzuela CF, Harris RA. Effects of ethanol on recombinant human neuronal nicotinic acetylcholine receptors expressed in *Xenopus* oocytes. J Pharmacol Exp Ther 1999;289:774–80.
[7] Carmelli D, Swan GE, Robinette D, Fabsitz RR. Heritability of substance use in the NAS–NRC Twin Registry. Acta Genet Med Gemellol (Roma) 1990;39:91–8.
[8] Champtiaux N, Han ZY, Bessis A, Rossi FM, Zoli M, Marubio L, et al. Distribution and pharmacology of alpha6-containing nicotinic acetylcholine receptors analyzed with mutant mice. J Neurosci 2002;22:1208–17.
[9] Dawson DA. Drinking as a risk factor for sustained smoking. Drug Alcohol Depend 2000;59:235–49.
[10] de Fiebre CM, Medhurst LJ, Collins AC. Nicotine response and nicotinic receptors in long-sleep and short-sleep mice. Alcohol 1987;4:493–501.
[11] de Fiebre CM, Romm E, Collins JT, Draski LJ, Deitrich RA, Collins AC. Responses to cholinergic agonists of rats selectively bred for differential sensitivity to ethanol. Alcohol Clin Exp Res 1991;15:270–6.
[12] de Fiebre CM, Collins AC. Classical genetic analyses of responses to nicotine and ethanol in crosses derived from long- and short-sleep mice. J Pharmacol Exp Ther 1992;261:173–80.
[13] de Fiebre NC, Dawson Jr R, de Fiebre CM. The selectively bred high alcohol sensitivity (HAS) and low alcohol sensitivity (LAS) rats differ in sensitivity to nicotine. Alcohol Clin Exp Res 2002;26:765–72.
[14] Dobelis P, Marks MJ, Whiteaker P, Balogh SA, Collins AC, Stitzel JA. A polymorphism in the mouse neuronal alpha4 nicotinic receptor subunit results in an alteration in receptor function. Mol Pharmacol 2002;62:334–42.
[15] Gill K, Desaulniers N, Desjardins P, Lake K. Alcohol preference in AXB/BXA recombinant inbred mice: gender differences and gender-specific quantitative trait loci. Mamm Genome 1998;9:929–35.
[16] Gordon TL, Meehan SM, Schechter MD. Differential effects of nicotine but not cathinone on motor activity of P and NP rats. Pharmacol Biochem Behav 1993;44:657–9.
[17] Gordon TL, Meehan SM, Schechter MD. P and NP rats respond differently to the discriminative stimulus effects of nicotine. Pharmacol Biochem Behav 1993;45:305–8.
[18] Heath AC, Cates R, Martin NG, Meyer J, Hewitt JK, Neale MC, et al. Genetic contribution to risk of smoking initiation: comparisons across birth cohorts and across cultures. J Subst Abuse 1993;5:221–46.
[19] Heath AC, Martin NG. Genetic models for the natural history of smoking: evidence for a genetic influence on smoking persistence. Addict Behav 1993;18:19–34.
[20] Hettema JM, Corey LA, Kendler KS. A multivariate genetic analysis of the use of tobacco, alcohol, and caffeine in a population based sample of male and female twins. Drug Alcohol Depend 1999;57:69–78.
[21] Istvan J, Matarazzo JD. Tobacco, alcohol, and caffeine use: a review of their interrelationships. Psychol Bull 1984;95:301–26.
[22] Kandel D, Chen K, Warner LA, Kessler RC, Grant B. Prevalence and demographic correlates of symptoms of last year dependence on alcohol, nicotine, marijuana and cocaine in the U.S. population. Drug Alcohol Depend 1997;44:11–29.
[23] Katner SN, McBride WJ, Lumeng L, Li TK, Murphy JM. Effects of cholinergic agents on locomotor activity of P and NP rats. Alcohol Clin Exp Res 1996;20:1004–10.
[24] Kim H, Flanagin BA, Qin C, Macdonald RL, Stitzel JA. The mouse Chrna4 A529T polymorphism alters the ratio of high to low affinity α4β2 nAChRs. Neurpharmacology 2003;45:345–54.
[25] Lindstrom J. Nicotinic acetylcholine receptors in health and disease. Mol Neurobiol 1997;15:193–222.
[26] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–75.
[27] Marks MJ, Romm E, Campbell SM, Collins AC. Variation of nicotinic binding sites among inbred strains. Pharmacol Biochem Behav 1989:33.
[28] Marks MJ, Grady SR, Collins AC. Downregulation of nicotinic receptor function after chronic nicotine infusion. J Pharmacol Exp Ther 1993;266:1268–76.
[29] Meliska CJ, Bartke A, Vandergriff JL, Jensen RA. Ethanol and nicotine consumption and preference in transgenic mice overexpressing the bovine growth hormone gene. Pharmacol Biochem Behav 1995;50:563–70.
[30] Meliska CJ, Bartke A, McGlacken G, Jensen RA. Ethanol, nicotine, amphetamine, and aspartame consumption and preferences in C57BL/6 and DBA/2 mice. Pharmacol Biochem Behav 1995;50: 619–26.
[31] Miner LL, Collins AC. Strain comparison of nicotine-induced seizure sensitivity and nicotinic receptors. Pharmacol Biochem Behav 1989;33:469–75.
[32] Robinson SF, Marks MJ, Collins AC. Inbred mouse strains vary in oral self-selection of nicotine. Psychopharmacology (Berl) 1996;124:332–9.
[33] Ryan LJ, Barr JE, Sanders B, Sharpless SK. Electrophysiological responses to ethanol, pentobarbital, and nicotine in mice genetically selected for differential sensitivity to ethanol. J Comp Physiol Psychol 1979;93:1035–52.
[34] Salminen O, Murphy KL, McIntosh JM, Drago J, Marks MJ, Collins AC, et al. Subunit composition and pharmacology of two classes

of striatal presynaptic nicotinic acetylcholine receptors mediating dopamine release in mice. Mol Pharmacol 2004;65:1526–35.
[35] Söderpalm B, Ericson M, Olausson P, Blomqvist O, Engel JA. Nicotinic mechanisms involved in the dopamine activating and reinforcing properties of ethanol. Behav Brain Res 2000;113: 85–96.
[36] Stitzel JA, Jimenez M, Marks MJ, Tritto T, Collins AC. Potential role of the alpha4 and alpha6 nicotinic receptor subunits in regulating nicotine-induced seizures. J Pharmacol Exp Ther 2000;293:67–74.
[37] Stitzel JA, Dobelis P, Jimenez M, Collins AC. Long sleep and short sleep mice differ in nicotine-stimulated 86Rb+ efflux and alpha4 nicotinic receptor subunit cDNA sequence. Pharmacogenetics 2001;11:331–9.
[38] Swan GE, Carmelli D, Rosenman RH, Fabsitz RR, Christian JC. Smoking and alcohol consumption in adult male twins: genetic heritability and shared environmental influences. J Subst Abuse 1990;2:39–50.
[39] Tizabi Y, Getachew B, Davila-Garcia M, Taylor RE. Alcohol preference: association with reduced striatal nicotinic receptors. Alcohol Alcohol 2001;36:318–22.
[40] Todte K, Tselis N, Dadmarz M, Golden G, Ferraro T, Berrettini WH, et al. Effects of strain, behavior and age on the self-administration of ethanol, nicotine, cocaine and morphine by two rat strains. Neuropsychobiology 2001;44:150–5.
[41] Tritto T, Marley RJ, Bastidas D, Stitzel JA, Collins AC. Potential regulation of nicotine and ethanol actions by alpha4-containing nicotinic receptors. Alcohol 2001;24:69–78.
[42] Tritto T, Stitzel JA, Marks MJ, Romm E, Collins AC. Variability in response to nicotine in the LSxSS RI strains: potential role of polymorphisms in alpha4 and alpha6 nicotinic receptor genes. Pharmacogenetics 2002;12:197–208.
[43] True WR, Xian H, Scherrer JF, Madden PA, Bucholz KK, Heath AC, et al. Common genetic vulnerability for nicotine and alcohol dependence in men. Arch Gen Psychiatry 1999;56:655–61.
[44] Yang X, Criswell HE, Breese GR. Action of ethanol on responses to nicotine from cerebellar interneurons and medial septal neurons: relationship to methyllycaconitine inhibition of nicotine responses. Alcohol Clin Exp Res 1999;23:983–90.
[45] Zoli M, Moretti M, Zanardi A, McIntosh JM, Clementi F, Gotti C. Identification of the nicotinic receptor subtypes expressed on dopaminergic terminals in the rat striatum. J Neurosci 2002;22: 8785–9.
[46] Zuo Y, Aistrup GL, Marszalec W, Gillespie A, Chavez-Noriega LE, Yeh JZ, et al. Dual action of *n*-alcohols on neuronal nicotinic acetylcholine receptors. Mol Pharmacol 2001;60:700–11.