

Modulation of Nicotine but Not Ethanol Preference by the Mouse *Chrna4* A529T Polymorphism

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Available evidence indicates that common genes influence alcohol and tobacco abuse in humans. The studies reported here used mouse models to evaluate the hypothesis that genetically determined variability in the $\alpha 4\beta 2^*$ nicotinic receptor modulates genetically determined variability in the intake of both nicotine and alcohol. Data obtained with inbred mouse strains suggested an association between a polymorphism in the mouse $\alpha 4$ nAChR subunit gene, *Chrna4*, and variability in nicotine and ethanol preference. These associations were assessed in F2 animals derived by crossing C57BL/6 $\beta 2^{-/-}$ mice and A/J mice. The results obtained by the authors indicate that the polymorphism in *Chrna4* plays an important role in modulating variability in oral nicotine intake but is linked to a gene that regulates alcohol intake.

Nicotine and alcohol are commonly coabused by humans. Laboratory studies demonstrate that people will consume more alcohol when cigarettes are accessible (Perkins, Fonte, & Grobe, 2000), and tobacco use increases when ethanol is accessible (Mitchell, de Wit, & Zacny, 1995). Genetic studies indicate that common genes may influence alcoholism and tobacco (nicotine) abuse (reviewed in Madden & Heath, 2002). For example, a region in human chromosome 20 that contains the gene encoding the $\alpha 4$ subunit of the neuronal nicotinic acetylcholine receptor (nAChR; Bessis, Simon-Chazottes, Devillers-Thiery, Guenet, & Changeux, 1990) has been associated with some aspects of alcoholism (Schuckit et al., 2001), and polymorphisms in the $\alpha 4$ subunit gene recently have been demonstrated to be associated with tobacco use (Feng et al., 2004). Moreover, in vitro studies have shown that ethanol directly increases the maximal agonist-induced ion flux through the $\alpha 4\beta 2$ nAChR (Aistrup, Marszalec, & Narahashi, 1999; Cardoso et al., 1999). These findings suggest that nicotine and ethanol may exert common effects through the $\alpha 4\beta 2$ nAChR complex.

The nAChR is the primary mediator of nicotine's effects in the central nervous system. This pentameric ligand-gated ion channel is formed from a variety of α ($\alpha 2$ – $\alpha 10$) and β ($\beta 2$ – $\beta 4$) subunits.

The predominant nAChR subtype in the brain contains both $\alpha 4$ and $\beta 2$ subunits (Guo & Chiappinelli, 2002; Role & Berg, 1996). These two subunits are critical components of the high-affinity nicotine binding site. Mice that lack either the $\beta 2$ (Picciotto et al., 1995) or $\alpha 4$ (Marubio et al., 1999) subunits do not exhibit high-affinity nicotine binding in any brain region. In addition, $\beta 2^{-/-}$ mice do not self-administer nicotine whereas $\beta +/+$ mice do (Picciotto et al., 1998).

Previous work in our laboratory has identified a single nucleotide polymorphism in *Chrna4* (Stitzel, Jimenez, Marks, Tritto, & Collins, 2000). The polymorphism results in an alanine (A) or a threonine (T) residue at amino acid position 529 in the second intracellular loop of the $\alpha 4$ protein (Kim, Flanagan, Qin, Macdonald, & Stitzel, 2003) and has been associated with differences in a number of neurochemical and behavioral responses to nicotine and alcohol. The *Chrna4* polymorphism influences receptor function as assessed by an ion flux assay using mouse brain synaptosomes (Butt et al., 2003; Dobelis et al., 2002) and as evaluated using electrophysiological recording methods in a heterologous expression system (Kim et al., 2003). Furthermore, the enhancement of nAChR function by ethanol appears to be determined by A529T genotype; that is, the enhancement of agonist-induced ion flux is greater when the A529 variant is present (Butt et al., 2003). Sensitivity to several responses to both nicotine and alcohol measured in vivo is also associated with the *Chrna4* polymorphism. The polymorphism appears to influence variability in nicotine-induced seizures (Stitzel et al., 2000), ethanol withdrawal seizures (Butt, King, Stitzel, & Collins, 2004), the effect of both drugs on locomotor activity (Tritto, Marley, Bastidas, Stitzel, & Collins, 2001; Tritto, Stitzel, Marks, Romm, & Collins, 2002), and alcohol-induced depression of acoustic startle (Owens et al., 2003).

All of the associations between the polymorphism in *Chrna4* and behavioral responses to nicotine and alcohol that we have detected are provocative. However, additional studies are required to confirm that the A529T polymorphism, rather than a linked gene, is responsible for the associations that we have observed. To evaluate the relationship between the *Chrna4* polymorphism and behaviors, we used an F2 intercross derived from nAChR $\beta 2$ null

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mutant mice (C57BL/6 background; B6 ^{$\beta 2^{-/-}$}) and A/J mice. Mice that lack *Chrb2* do not express $\alpha 4\beta 2^*$ nAChRs (Marubio et al., 1999; Picciotto et al., 1995). All, or nearly all, nAChRs that contain the $\alpha 4$ subunit also include the $\beta 2$ subunit. Any association between the *A529T* polymorphism that is lost in the $\beta 2^{-/-}$ mice provides compelling evidence supporting the argument that $\alpha 4\beta 2^*$ nAChRs modulate the phenotype being studied. This result was obtained in a study that evaluated the association between the *A529T* polymorphism and sensitivity to handling-induced convulsion (HIC) measured after acute ethanol treatment (Butt et al., 2004). The B6 ^{$\beta 2^{+/+}$} A F2 mice that were homozygous for the *A529T* allele had significantly more severe withdrawal symptoms than *T529* homozygous B6 ^{$\beta 2^{+/+}$} A F2 mice. In contrast, *A529T* differences in ethanol HIC were not found in B6 ^{$\beta 2^{-/-}$} A F2 mice. These findings indicate that the association of the *A529T* polymorphism with ethanol HIC is conferred by the polymorphism itself, not by genes linked to *Chrna4*.

Genetic factors play an important role in regulating the oral intake of both nicotine (Robinson, Marks, & Collins, 1996) and alcohol (Belknap, Crabbe, & Young, 1993; Gill, Desaulniers, Desjardins, & Lake, 1998) in mice. Virtually nothing is known about the genetic regulation of nicotine intake except that intake is negatively correlated with sensitivity to nicotine-induced seizures (Robinson et al., 1996). Because sensitivity to nicotine-induced seizures is associated with the *A529T* polymorphism (Stitzel et al., 2000), the experiments reported here investigated the potential role of the *A529T Chrna4* polymorphism in regulating nicotine intake. Oral ethanol intake was also measured since ethanol withdrawal severity, which is also associated with the *A529T Chrna4* polymorphism (Butt et al., 2004), is inversely correlated with ethanol intake (Metten et al., 1998). In addition, alcohol intake has recently been shown to be positively correlated with nicotine intake in mice (Li, Karadesh, Jenkins, & Stitzel, in press). These studies used data obtained with inbred strains as well as the B6 ^{$\beta 2^{-/-}$} A F2 intercross mice. The results obtained suggest that the *A529T* polymorphism contributes to individual differences in nicotine preference. However, the association between the *A529T Chrna4* polymorphism and ethanol preference likely is a result of genes linked to *Chrna4*.

Method

Mice

Male mice from 14 strains (129/SvEv, A/J, AKR/J, BALB/cByJ, BUB/BnJ, C3H/Ibg, C57BL/6J, C57BL/10J, C57BR/cdJ, C58/J, CBA/J, DBA/1J, DBA/2J, and RIIS/J) were used in the nicotine consumption studies. The strains were selected in an attempt to obtain nearly equal numbers of strains that express the *A529* and *T529* variants of $\alpha 4$ as identified by Dobelis et al. (2002). Half of the mouse strains used were produced from breeder mice maintained at the Institute for Behavioral Genetics, University of Colorado at Boulder. The AKR/J, C57BL/10J, C57BR/cdJ, C58/J, CBA/J, DBA/1J, and RIIS/J strains were purchased directly from Jackson Laboratories (Bar Harbor, ME). An F2 intercross was also generated to study both nicotine and ethanol preference (see *Development of F2 Mice* for details).

All mice were weaned and separated by sex when they were 25 days old. They were then housed in groups of 5 to a cage and maintained on a 12-hr light–dark cycle (lights on from 0700 to 1900). All mice were given unlimited access to food (Harlan Teklad Rodent Diet; Madison, WI) and water. Mice were 60–120 days old when used. All animal care and experimental procedures were approved by and performed in accordance

with the guidelines of the Animal Care and Utilization Committee of the University of Colorado at Boulder.

Development of F2 Mice

An F2 intercross was generated by crossing the A/J strain with C57BL/6 mice containing a null mutation (“knockout”) of the $\beta 2$ nAChR subunit gene (*Chrb2*; Picciotto et al., 1995). A/J mice express the *A529* variant of the $\alpha 4$ polymorphism, whereas C56BL/6J mice express the *T529* variant (Dobelis et al., 2002). The $\beta 2$ subunit gene deletion had been bred for eight generations onto the C57BL/6J background. Six different parental matings were made by crossing A/J mice with C57BL/6 ^{$\beta 2^{-/-}$} mice. Six families of F1 mice, which were made by crossing F1 mice from different parental matings, were then used to produce the B6 ^{$\beta 2^{-/-}$} A F2 progeny. Both male and female mice from this F2 intercross were used in the two-bottle assays of nicotine or ethanol preference (see *Nicotine Preference Studies* and *Ethanol Preference Studies*). B6 ^{$\beta 2^{+/+}$} A F2 mice were not used in this study.

Genotyping

We used tail clippings (~ 1 cm) to genotype every F2 mouse. DNA was extracted from the clippings with a Qiagen DNEasy Tissue Kit (Valencia, CA). $\beta 2$ genotypes were determined by polymerase chain reaction (PCR) with oligonucleotide probes specific for the *Chrb2* sequence. The *Chrna4 A529T* genotype was determined by PCR amplification with oligonucleotide probes specific for a region of *Chrna4* that flanks the *A529T* polymorphism followed by digestion with the restriction endonuclease *StuI* (New England Biolabs; Beverly, MA; Dobelis et al., 2002). The gene products were electrophoresed on 1.5% agarose gels and stained with ethidium bromide. Two independent observers then scored the genotypes.

Nicotine Preference Studies

Nicotine preference and consumption were determined with two- or four-bottle choice assays. Bottles were constructed from 15-ml (four-bottle choice) or 50-ml (two-bottle choice) centrifuge tubes that were fitted with singly perforated rubber stoppers. A metal sipper tube containing a ball-bearing valve was then inserted through the perforation. Mice were weighed at initiation and completion of the experiments. During the experiments, they were individually housed and spent the first 4 days of each study acclimating to their new conditions. During this acclimation period, the mice were given a choice between a bottle containing tap water or an empty bottle. In the two-bottle assays, the volume of fluid consumed was recorded daily at 1600, and the bottles were switched from one side of the cage to the other to reduce the effects of side preference. After the acclimation period, the empty bottle was filled with a nicotine (25 $\mu\text{g/ml}$) solution made in tap water. The nicotine concentration was then increased every 4 days. After 4 days with the 25 $\mu\text{g/ml}$ solution, the nicotine solution was changed to 50 $\mu\text{g/ml}$ and finally to 100 $\mu\text{g/ml}$. The daily bottle switching and recording of water and nicotine consumption continued throughout the 16-day assay. Three empty “dummy” cages were also constructed for each preference condition in order to account for the leakage of solution from the bottles. Only the B6 ^{$\beta 2^{-/-}$} A F2 hybrids were exposed to the two-bottle nicotine preference studies. All other nicotine studies used the four-bottle choice assays. In the 12-day, four-bottle choice assays, the mice were given simultaneous access to all three nicotine concentrations (25 $\mu\text{g/ml}$, 50 $\mu\text{g/ml}$, and 100 $\mu\text{g/ml}$) and water. The positions of the four bottles were rotated daily along with the recording of the volumes consumed of each solution.

A possible confound of measuring oral intake as a potential measure of the reinforcing effects of drugs is the taste of those drugs. Taste factors may interact with pharmacological actions of the drugs to influence drug choice; that is, an animal might choose to drink, or to avoid drinking, a drug

solution because it has a pleasant or obnoxious taste, respectively. An appropriate control for these experiments might be an agent with identical taste to the test drug that has little or no pharmacological activity. No such compound has been identified for alcohol, but D-nicotine, which is approximately 20 times less potent than the L-isomer (Marks, Robinson, & Collins, 1996; Shimada, Iizuka, Kawaguchi, & Yanagita, 1984), might serve as an appropriate control. The value of D-nicotine as a control is enhanced if D- and L-nicotine taste the same to a mouse, which, obviously, is difficult to determine. The data obtained with D- and L-nicotine should be similar if taste is the principal determinant that influences drug choice. However, if pharmacological factors are the principal determinants that influence intake, differences between the D- and L-nicotine isomers should be seen. Given that the preference of mouse strains for L-nicotine generally ranges from indifference (equal volumes of water and drug solution are consumed) to almost total rejection (Adriani, Macri, Pacifici, & Laviola, 2002; Robinson et al., 1996) we suggest that mice will avoid D-nicotine to a lesser degree than L-nicotine if pharmacological factors are influencing drug choice. Therefore, some nicotine preference studies evaluated the possible effects of taste by using the less potent D-nicotine isomer in the drug solution (Marks et al., 1996; Shimada et al., 1984). Experiments with D-nicotine were performed separately from those with L-nicotine. Both the L- and D-nicotine were used in the free base form and were purchased from Sigma Chemical (St. Louis, MO).

Ethanol Preference Studies

Ethanol preference and consumption were determined in the same manner as the two-bottle nicotine choice assays except that the ethanol concentrations used were 3%, 7%, and 10% (vol/vol) in tap water.

Data Analysis

Absolute consumption and preference for the drug solution were calculated in several steps. First, the amounts of each solution consumed per day were averaged over the periods that the mice were exposed to them (i.e., 4 days). The average leakage of each solution was then subtracted. Preference was expressed as a ratio of the amount of drug solution consumed divided by the amount of total fluid (drug solution plus tap water) consumed. Absolute consumption was expressed as the mass of drug consumed (milligrams of nicotine or grams of ethanol) per average mass of the mouse per day.

The data were initially tested for normal distributions with SPSS 12.0 (SPSS, Chicago, IL). Most of the data were not distributed normally. Therefore, all the data were normalized with a square root transform. Testing for significant differences was performed with unpaired, two-tailed *t* tests ($p < .05$; GraphPad Prism 3.0, GraphPad Software, Inc., San Diego, CA) when only two distinct data sets were involved. SPSS 12.0 was used to run analyses of variance (ANOVA) on data sets that involved multiple independent variables with significance levels set at $p < .05$. All power values are reported as $(1 - \beta)$, the difference between 1 and the probability of Type II error, in each analysis.

Data from previously published ethanol preference studies were also analyzed with regard to the *A529T* polymorphism (Belknap et al., 1993; Gill et al., 1998; Rodriguez, Plomin, Blizard, Jones, & McClearn, 1994, 1995). For each of these studies, the averages of the ethanol measures were grouped according to the *A529T* genotype of the mice involved, and the differences between the groups were analyzed with unpaired *t* tests or two-way ANOVAs (if multiple drug concentrations were used). These meta-analyses evaluated ethanol consumption in 15 inbred strains (Belknap et al., 1993) 29 AxB/BxA recombinant inbred strains (Gill et al., 1998), and 23 BxD recombinant inbred strains (Rodriguez et al., 1994, 1995). The *A529T* genotypes of the inbred strains are reported in Dobelis et al. (2002). The *A529T* genotypes of the AxB/BxA and BxD recombinant inbred strains were determined with the methods described above on DNA sam-

ples supplied by Drs. Katherine Gill (McGill University) and John Crabbe (Oregon Health Sciences Center), respectively. Since the genetic markers *D2Mit200* and *D2Mit74* (both at 107 centimorgans on chromosome 2; Mouse Genome Database [MGD], 2004) are in close proximity to the gene for the $\alpha 4$ nAChR (108 centimorgans on chromosome 2; Bessis et al., 1990) we also performed a similar analysis of the previously published recombinant inbred strain data with regard to genotypes at the two markers.

Results

L-Nicotine Consumption in Inbred Mouse Strains

Figure 1 shows the results of the 14-strain, four-bottle nicotine consumption study that was performed with male mice. Two-way ANOVA (Genotype \times Concentration) detected a significant effect of *A529T* genotype on nicotine consumption. The effect of strain was also robust: two-way ANOVA (Strain \times Concentration), $F(13, 225) = 8.25, p < .001, (1 - \beta) = 1.000$. Concentration did not have a main effect or any interactions. A three-way ANOVA (Strain \times Genotype \times Concentration) was not possible because genotype was a subset of strain. The average daily consumption values of all 14 strains for each nicotine concentration are found in Table 1.

D-Nicotine Consumption in Five Inbred Strains

Figure 2 demonstrates that all five mouse strains that were tested in the four-bottle choice assay exhibited significantly higher consumption of the physiologically less potent D-nicotine enantiomer than the L-nicotine enantiomer. A two-way ANOVA (Genotype \times Concentration) of the D-nicotine data indicated that D-nicotine consumption was not affected by *A529T* genotype. In another two-way ANOVA (Strain \times Concentration), D-nicotine consumption was significantly affected by strain, $F(4, 45) = 6.14, p < .001, (1 - \beta) = 0.978$, and concentration, $F(2, 45) = 57.60, p < .001$,

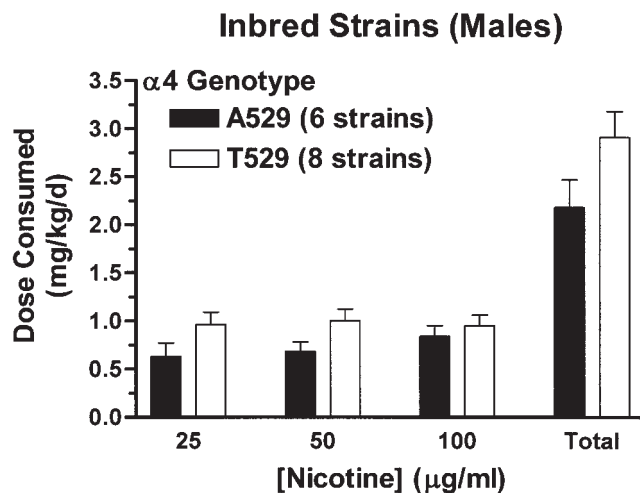


Figure 1. Effect of *A529T* genotype on nicotine consumption in inbred mouse strains. This 14-strain study of nicotine consumption demonstrated that mice with the *A529* genotype consumed significantly less nicotine than mice with the *T529* genotype, two-way ANOVA (Genotype \times Concentration), $F(1, 261) = 7.68, p = .006, (1 - \beta) = 0.788$. Error bars represent standard error of the mean. d = day.

Table 1
Genotype and Average Daily Nicotine Consumption of the Inbred Strains Studied

Strain	n	Chrna4 genotype ^a	Nicotine consumption (mg/kg/day) ± SEM, by nicotine dosage (µg/ml)		
			25	50	100
129/SvEv	6	T529	0.73 ± 0.24	0.38 ± 0.15	0.29 ± 0.10
A/J	11	A529	0.47 ± 0.17	0.52 ± 0.11	0.78 ± 0.27
AKR/J	6	A529	1.73 ± 0.68	1.57 ± 0.36	1.47 ± 0.25
BALB/cByJ	6	T529	0.69 ± 0.22	1.20 ± 0.58	0.72 ± 0.22
BUB/BnJ	6	T529	0.64 ± 0.13	1.08 ± 0.20	1.06 ± 0.22
C3H/lbg	5	A529	0.46 ± 0.14	0.22 ± 0.07	0.16 ± 0.09
C57BL/6J	11	T529	2.00 ± 0.38	1.39 ± 0.20	1.64 ± 0.28
C57BL/10J	6	T529	0.65 ± 0.14	0.76 ± 0.34	0.75 ± 0.19
C57BR/cdJ	5	T529	1.18 ± 0.44	1.34 ± 0.34	1.60 ± 0.39
C58/J	6	T529	0.47 ± 0.11	1.00 ± 0.60	0.79 ± 0.18
CBA/J	6	T529	0.52 ± 0.21	0.55 ± 0.13	0.32 ± 0.19
DBA/1J	6	A529	0.45 ± 0.13	0.71 ± 0.16	0.86 ± 0.19
DBA/2J	6	A529	0.26 ± 0.07	0.58 ± 0.23	0.73 ± 0.15
RIIIS/J	6	A529	0.44 ± 0.13	0.48 ± 0.11	0.92 ± 0.36

^a Dobelis et al. (2002).

(1-β) = 1.000, and a significant interaction occurred between concentration and strain, $F(8, 45) = 5.37, p < .001, (1-\beta) = 0.997$. Three-way ANOVA of the five-strain D-/L-nicotine data set (Genotype × Concentration × Enantiomer) found significant effects of A529T genotype, $F(1, 156) = 24.60, p < .001, (1-\beta) = 0.999$; enantiomer, $F(1, 156) = 122.20, p < .001, (1-\beta) = 1.000$; and concentration, $F(2, 156) = 24.80, p < .001, (1-\beta) = 1.000$, and a significant interaction between concentration and enantiomer, $F(2, 156) = 19.20, p < .001, (1-\beta) = 1.000$. A three-way ANOVA comparing strain, concentration, and enantiomer effects on nicotine consumption yielded similar results. However, this particular analysis also detected a significant effect of strain, $F(4, 138) = 9.17, p < .001, (1-\beta) = 0.999$, and three additional interactions: Strain × Enantiomer, $F(4, 138) = 4.99, p = .001, (1-\beta) = 0.958$; Strain × Concentration, $F(8, 138) = 2.85, p = .006, (1-\beta) = 0.937$; and Concentration × Enantiomer × Strain, $F(8, 138) = 2.96, p = .004, (1-\beta) = 0.946$. Again, genotype and strain could not be used in the same analysis because genotype was a subset of strain. Two-way ANOVAs (Concentration × Enantiomer) within each strain indicated significant effects of enantiomer (i.e., each strain consumed more D-nicotine than L-nicotine). In addition, all but the RIIIS strain showed significant effects of concentration and significant interactions between concentration and enantiomer: A/J concentration, $F(2, 30) = 14.80, p < .001, (1-\beta) = 0.998$; A/J interaction, $F(2, 30) = 9.89, p = .001, (1-\beta) = 0.973$; DBA/1J concentration, $F(2, 21) = 13.20, p < .001, (1-\beta) = 0.993$; DBA/1J interaction, $F(2, 21) = 7.24, p = .004, (1-\beta) = 0.896$; C57BL6J concentration, $F(2, 42) = 4.30, p = .020, (1-\beta) = 0.718$; C57BL6J interaction, $F(2, 42) = 10.30, p < .001, (1-\beta) = 0.981$; C57BR/cdJ concentration, $F(2, 18) = 15.70, p < .001, (1-\beta) = 0.998$; C57BR/cdJ interaction, $F(2, 18) = 9.90, p = .001, (1-\beta) = 0.963$. To simplify, we used an unpaired, two-tailed *t* test and detected a significant effect of A529T genotype on total consumption of L-nicotine but not total consumption of D-nicotine (see Figure 2F).

F2 Analysis of Nicotine Preference and Consumption

Figure 3 demonstrates an important role for A529T genotype in nicotine preference and consumption in the B6^{β2-/-}A F2 intercross. A three-way ANOVA (Genotype × Concentration × Sex) on the data obtained from B6^{β2+/+}A F2 mice (see Figure 3A) indicated significant effects of genotype and concentration, $F(2, 138) = 15.50, p < .001, (1-\beta) = 0.999$, on nicotine preference. The results for nicotine consumption (dose consumed) in the B6^{β2+/+}A F2 mice (see Figure 3B) were largely the same as those for nicotine preference. A three-way ANOVA on these consumption data detected significant effects of genotype and concentration, $F(2, 138) = 4.57, p = .012, (1-\beta) = 0.768$.

Figures 3C and 3D demonstrate that A529T genotype had no effect on nicotine preference and consumption in F2 mice that lack *Chrb2*. Nicotine concentration was the only variable detected as having a significant effect on nicotine preference, $F(2, 105) = 5.32, p = .006, (1-\beta) = 0.829$ (see Figure 3C), and nicotine consumption, $F(2, 105) = 5.13, p = .007, (1-\beta) = 0.814$ (see Figure 3D), in a three-way ANOVA of the data obtained from B6^{β2-/-}A F2 mice.

Ethanol Preference in Inbred and AxB/BxA Recombinant Inbred Mouse Strains

Figure 4 illustrates that when the ethanol preference ratios of male mice from a number of mouse strains were averaged according to their A529T genotype, a significant association was detected between the *Chrna4* polymorphism and ethanol preference. Figure 4A is based on ethanol preference data reported by Belknap et al. (1993) across three ethanol doses (3%, 6%, and 10%). The five inbred mouse strains carrying the A529 variant of *Chrna4* include AKR/J (*n* = 9), C3H/HeJ (*n* = 10), A/HeJ (*n* = 9), DBA/1J (*n* = 8), and DBA/2J (*n* = 16). The 10 inbred strains carrying the T529

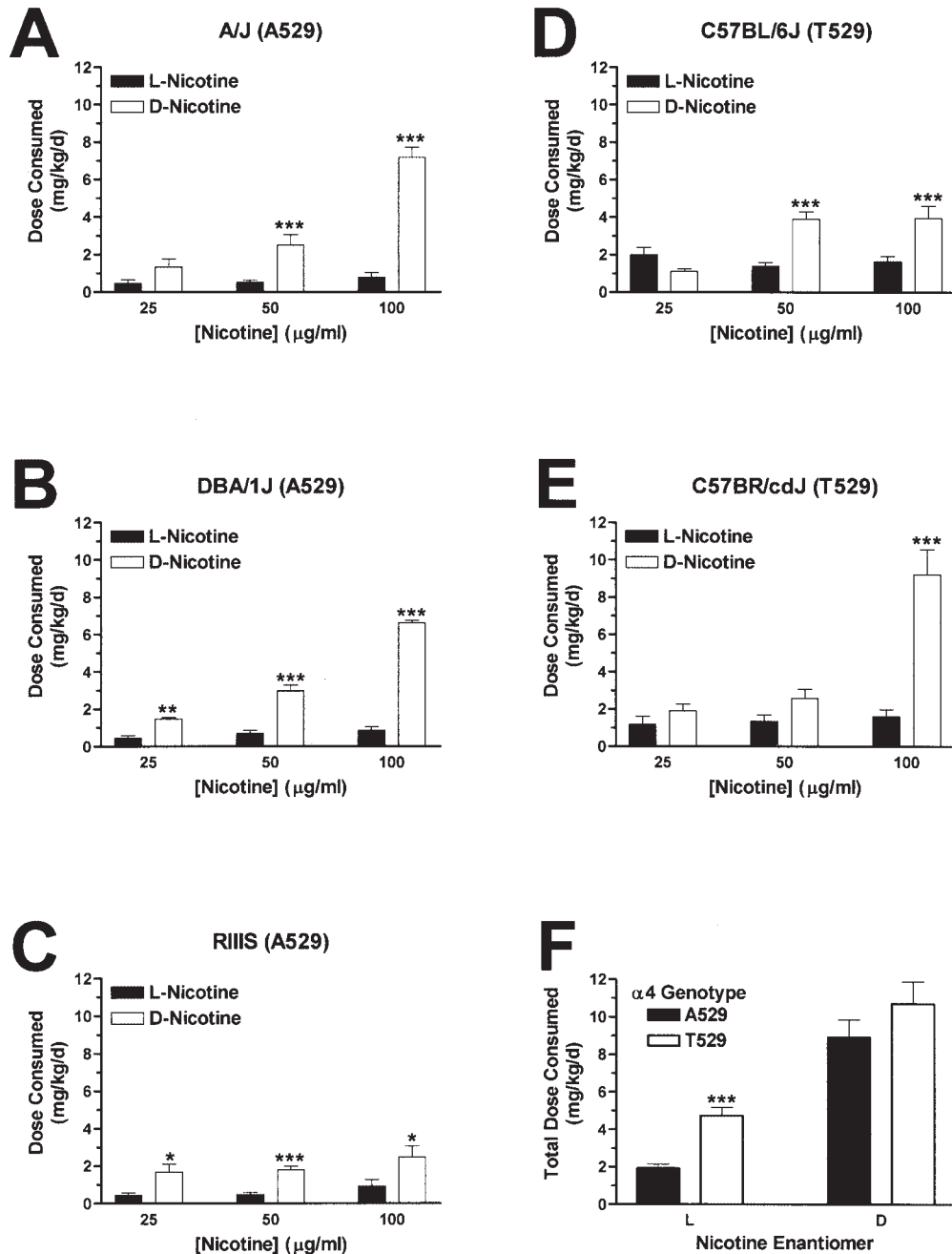
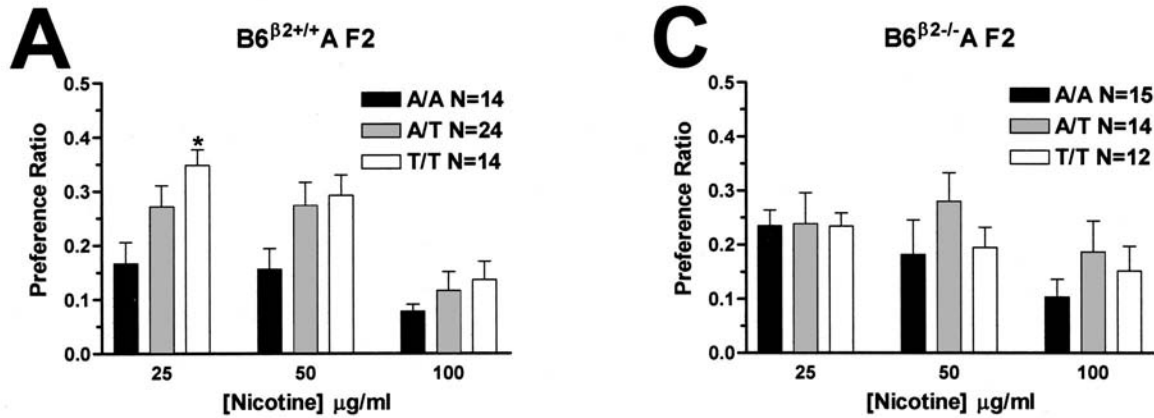


Figure 2. Taste aversion is not responsible for *A529T* effects on nicotine consumption. Three *A529*-containing strains (A, B, C) and two *T529*-containing strains (D, E) were studied for possible consumption differences between the physiologically active L-nicotine and its inactive enantiomer, D-nicotine. ANOVA (Enantiomer \times Concentration) indicated that all five strains ($N = 3-5$ per strain) exhibited significantly higher consumption of D-nicotine with the following statistics: *A/J* strain (A), $F(1, 30) = 59.20$, $p < .001$, $(1-\beta) = 1.000$; *DBA/1J* strain (B), $F(1, 21) = 75.20$, $p < .001$, $(1-\beta) = 1.000$; *RIIS* strain (C), $F(1, 27) = 22.50$, $p < .001$, $(1-\beta) = 0.995$; *C57BL/6J* strain (D), $F(1, 42) = 13.00$, $p = .001$, $(1-\beta) = 0.941$; *C57BR/cdJ* strain (E), $F(1, 18) = 36.00$, $p < .001$, $(1-\beta) = 1.000$. All strains except for *RIIS* also had significant effects of concentration and significant interactions between concentration and enantiomer (see *D-Nicotine Consumption in Five Inbred Strains* for details). Error bars represent standard error of the mean. * $p < .05$, ** $p < .01$, and *** $p < .001$, compared with appropriate L-nicotine data in unpaired, two-tailed *t* tests. F: Total L-nicotine consumption was significantly lower in the three strains with *A529* genotype than in the two strains with *T529* genotype, *** $p < .001$, $(1-\beta) = 0.999$. However, there was no *A529T* difference in total D-nicotine consumption. d = day.

Preference



Dose Consumed

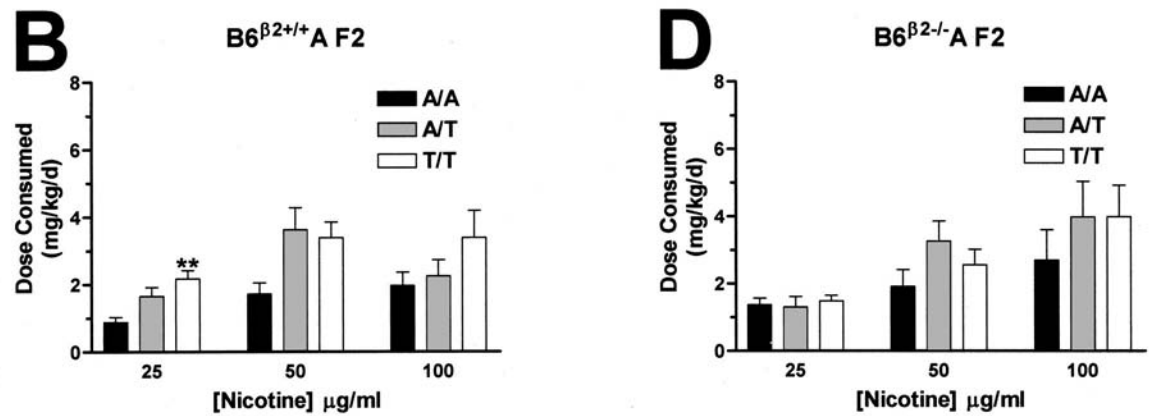


Figure 3. Nicotine preference and consumption in the $B6^{\beta 2-/-}$ A F2 intercross. Error bars represent standard error of the mean. A: A significant effect of *A529T* genotype on nicotine preference measured in $B6^{\beta 2+/+}$ A F2 mice was found in a three-way ANOVA (Genotype \times Concentration \times Sex), $F(2, 138) = 8.24, p < .001, (1-\beta) = 0.958$. B: Nicotine consumption in $B6^{\beta 2+/+}$ A F2 mice was also significantly associated with *A529T* genotype in a three-way ANOVA, $F(2, 138) = 5.75, p = .004, (1-\beta) = 0.861$. *A529T* genotype did not have a significant effect on preference (C) or consumption (D) in mice that lacked *Chrb2*. The sample sizes were as follows: $B6^{\beta 2+/+}$ A F2 A/A (9 males, 5 females), A/T (15 males, 9 females), T/T (9 males, 5 females), $B6^{\beta 2-/-}$ A F2 A/A (7 males, 8 females), A/T (5 males, 9 females), T/T (6 males, 6 females). d = day. * $p < .05$ and ** $p < .01$ compared with appropriate A/A data in Bonferroni post hoc tests.

variant include 129/J ($n = 10$), BALB/cJ ($n = 10$), C57BL/6J ($n = 16$), C57BR/cdJ ($n = 10$), C57L/J ($n = 9$), CBA/J ($n = 10$), CE/J ($n = 10$), PL/J ($n = 10$), SJL/J ($n = 10$), and SWR/J ($n = 10$). The *Chrna4* genotype of each of these inbred strains was reported by Kim et al. (2003). Strains carrying the *A529* variant had generally lower preference values for ethanol than the *T529*-containing strains. A two-way ANOVA (Genotype \times Concentration) indicated that the effect of genotype on the preference ratio approached significance ($p = .05$). Female mice were not used in Belknap et al. (1993). Figure 4B shows a stronger association of ethanol (10%) preference with the *Chrna4* polymorphism in the A \times B/B \times A recombinant inbred mouse strains. The data were

reported by Gill et al. (1998) and constitute the average preference values of 11 *A529*-containing strains and 18 *T529*-containing strains ($n = 14-30$ mice/strain; total $N = 659$). A two-way ANOVA (Genotype \times Sex) indicated a significant effect of genotype on preference, $F(1, 54) = 24.10, p < .001, (1-\beta) = 0.998$. There was no significant effect of sex or any significant interactions. An unpaired, two-tailed *t* test of the sex-pooled data indicated that recombinant inbred strains carrying the *A529* variant of *Chrna4* again had significantly lower preference values than strains with the *T529* variant. Table 2 contains the genotypes of the AxB/BxA recombinant inbred strains at *Chrna4* and *D2Mit74* (MGD, 2004).

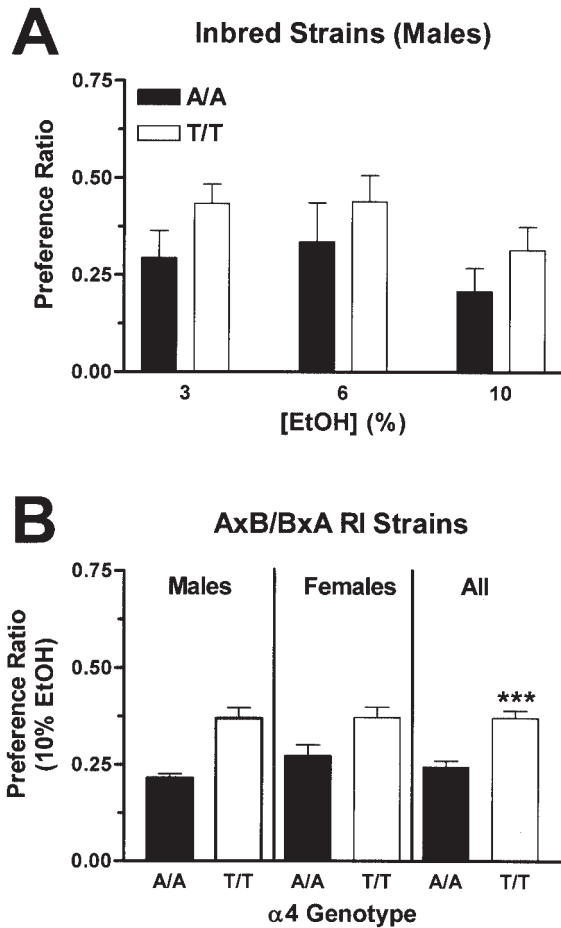


Figure 4. Ethanol (EtOH) preference ratios in inbred mouse strains and the AxB/BxA recombinant inbred mouse strains. Error bars represent standard error of the mean. **A:** Average ethanol preference ratios in 15 inbred mouse strains at three ethanol concentrations (Belknap et al., 1993). Strains carrying the *A529* variant of *Chrna4* ($n = 5$) generally had less preference for ethanol than mice with the *T529* variant ($n = 10$). The effect of genotype approached significance in a two-way ANOVA (Genotype \times Concentration), $F(1, 39) = 4.10$, $p = .050$, $(1-\beta) = 0.498$. Two-tailed unpaired t tests of the data at each ethanol concentration did not detect significant differences between the genotypes. **B:** Average preference for 10% ethanol in the AxB/BxA recombinant inbred (RI) strains (Gill et al., 1998). *A529*-containing strains had significantly lower preference for ethanol than *T529*-containing strains. *** $p < .001$, in an unpaired, two-tailed t test compared with the data obtained from *A/A* mice.

Ethanol Consumption in BxD Recombinant Inbred Mouse Strains

A trend was apparent in the BxD recombinant inbred mouse strains, but the association between the *Chrna4* polymorphism and ethanol consumption was not significant (see Figure 5). A two-way ANOVA (Genotype \times Sex) of the data reported by Rodriguez et al. (1994, 1995) indicated no significant effects of *A529T* genotype or sex on average daily ethanol consumption. An unpaired, two-tailed t test of the pooled male and female data also did not detect a statistical difference between the ethanol consumption of recombinant inbred strains carrying the *A529* variant of *Chrna4* and that

of strains with the *T529* variant. Table 2 contains the genotypes of the BxD recombinant inbred strains at *Chrna4* and *D2Mit200* (MGD, 2004).

F2 Analysis of Ethanol Preference and Consumption

Figure 6 shows that there was a significant association between *A529T* genotype on ethanol preference and consumption in $B6^{B2+/+}$ A F2 mice. A three-way ANOVA (Genotype \times Concentration \times Sex) of the data depicted in Figure 6A indicated a significant effect of genotype, $F(2, 123) = 11.2$, $p < .001$, $(1-\beta) = 0.991$, on ethanol preference. However, a significant interaction between sex and genotype was also found, $F(2, 123) = 4.01$, $p = .021$, $(1-\beta) = 0.707$. Two-way ANOVA (Genotype \times Concentration) of the male data (see Figure 6B) also showed a significant effect of genotype on ethanol preference. Although the female data (see Figure 6C) showed the same trend of *A529* mice having less preference for ethanol than *T529* mice, a two-way ANOVA indicated that the effect of genotype was not significant.

The results for ethanol consumption in the $B6^{B2+/+}$ A F2 mice (see Figures 6D–6F) were largely the same as those for ethanol preference. Three-way ANOVA on the data in Figure 6D showed significant effects of genotype, $F(2, 123) = 10.80$, $p < .001$, $(1-\beta) = 0.989$; concentration, $F(2, 123) = 11.70$, $p < .001$, $(1-\beta) = 0.993$; and interaction between sex and genotype, $F(1, 123) = 5.35$, $p = .006$, $(1-\beta) = 0.834$. Two-way ANOVA (Genotype \times Concentration) revealed that ethanol concentration had significant effects on ethanol consumption in both males, $F(2, 42) = 10.50$, $p < .001$, $(1-\beta) = 0.983$ (see Figure 6E), and females, $F(2, 81) = 7.39$, $p = .001$, $(1-\beta) = 0.932$ (see Figure 6F), separately. The effect of genotype was, again, significant in males but not in females.

Figure 7 demonstrates that the effects of *A529T* genotype on ethanol preference and consumption continued to be detected in F2 mice that lack *Chrb2*. A three-way ANOVA (Genotype \times Con-

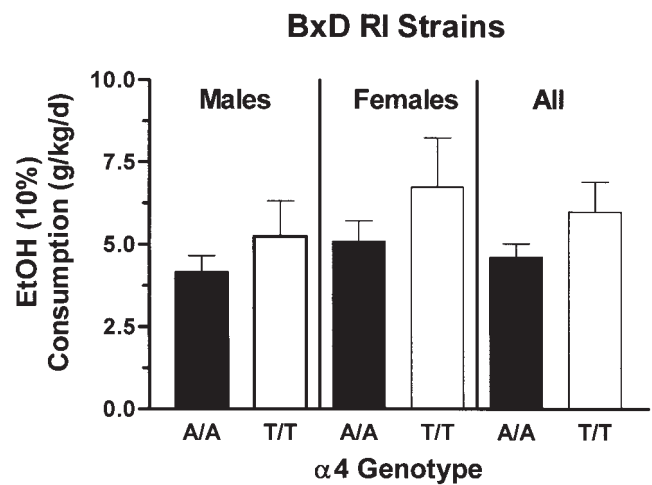


Figure 5. Ethanol (EtOH) consumption was not correlated with *A529T* genotype in the BxD recombinant inbred (RI) mouse strains (Rodriguez et al., 1994, 1995). An *A529T* trend was apparent, but no significant differences were detected. d = day. Error bars represent standard error of the mean.

Table 2
Genotypes of the AxB, BxA, and BxD Recombinant Inbred Strains at Chrna4, D2Mit74^a, and/or D2Mit200^a

AxB			BxA			BxD		
Strain	<i>Chrna4</i>	<i>D2Mit74</i>	Strain	<i>Chrna4</i>	<i>D2Mit74</i>	Strain	<i>Chrna4</i>	<i>D2Mit200</i>
A/J ^b	A529	A	C57BL/6J ^b	T529	B6	C57BL/6J ^b	T529	B6
1	T529	B6	2	T529	B6	DBA/2J ^b	A529	D2
2	A529	A	4	T529	B6	1	A529	D2
4	T529	A	7	A529	A	2	A529	D2
5	A529	A	8	A529	A	5	A529	D2
6	T529	B6	11	A529	A	6	T529	B6
8	A529	A	12	T529	A	8	T529	B6
10	T529	B6	13	T529	B6	9	A529	D2
11	A529	A	14	T529	B6	11	A529	D2
12	T529	B6	16	T529	B6	12	A529	D2
13	T529	B6	17	A529	A	13	A529	D2
15	T529	B6	24	T529	B6	14	T529	B6
18	T529	B6	25	A529	A	16	T529	B6
19	T529	B6				18	A529	D2
20	T529	B6				21	A529	D2
24	A529	A				22	T529	B6
						24	T529	B6
						25	T529	B6
						27	A529	D2
						28	A529	D2
						29	T529	B6
						31	A529	D2
						32	A529	D2

^a Both markers at 107 cM on chromosome 2 (Mouse Genome Database, 2004). ^b Dobelis et al. (2002).

centration × Sex) on the sex-pooled data depicted in Figure 7A detected a significant effect of genotype on ethanol preference with no main effect of or interaction with sex. A two-way ANOVA of the ethanol preference data with the sexes separated (see Figures 7B and 7C; for consistency with Figure 6) again showed significant effects of *A529T* genotype. Similar results were achieved when analyzing the ethanol consumption (see Figures 7D–7F) results in these $B6^{\beta 2-/-}$ A F2 mice. Genotype had a significant effect on consumption in the three-way ANOVA of the sex-pooled data (see Figure 7D). Ethanol concentration also had main effects on consumption in these analyses, $F(2, 96) = 11.00, p < .001, (1-\beta) = 0.990$. The effect of genotype approached significance in a two-way ANOVA of the male data (see Figure 7E), but the effect of ethanol concentration was still seen, $F(2, 48) = 3.32, p = .045, (1-\beta) = 0.601$. The significant effects of genotype and concentration, $F(2, 48) = 8.11, p = .001, (1-\beta) = 0.947$, were both maintained in the female data (see Figure 7F).

Discussion

The genetic strategies of inbred strain comparisons, meta-analysis of recombinant inbred strain data, F2 analysis, and null mutation were all used to test the hypothesis that $\alpha 4$ -containing nAChRs are involved in nicotine and ethanol preference. The data presented in this article indicate that the *A529T* polymorphism is an important factor that influences preference and consumption of nicotine, but the polymorphism's association with ethanol preference and consumption is probably a result of linkage. This finding is consistent with observations that the *A529T* polymorphism influences variability in some, but not all, behavioral effects pro-

duced by nicotine injection (Stitzel et al., 2000; Tritto et al., 2002) and some, but not all, effects produced by ethanol injection (Butt et al., 2004; Tritto et al., 2001).

Earlier work with a small sample size suggested that mouse strains with the *T529* genotype consumed higher doses of nicotine than mouse strains with the *A529* genotype (Robinson et al., 1996). Our 14-strain study of nicotine consumption provides additional data that support the postulate that the *A529* variant of *Chrna4* is associated with lower nicotine consumption in the inbred mouse strains. These findings are consistent with in vitro results that demonstrated that $\alpha 4$ -containing nAChRs with the *T529* variant of *Chrna4* are less sensitive to nicotine's activating effects than those with the *A529* variant of *Chrna4* (Butt et al., 2003; Dobelis et al., 2002).

One question that arises in nicotine preference studies is whether an observed difference in preference is due to the pharmacological effect of nicotine or to a difference in how the animals taste the drug. This question was addressed by the D-nicotine experiments. D-nicotine is approximately 20 times less potent than L-nicotine both in vitro (Marks et al., 1996) and in vivo (Shimada et al., 1984), but it does have the same chemical properties and presumably the same taste. All of the strains studied had significantly higher consumption of D-nicotine than L-nicotine, but there was no detectable effect of *A529T* genotype on D-nicotine consumption. Therefore, the association between *A529T* genotype and nicotine consumption is likely the result of the pharmacological effect of nicotine and not the taste of the drug. To our knowledge, there are no published reports of high-affinity nicotine binding in or on the tongue. Nicotinic receptors have been identified in the

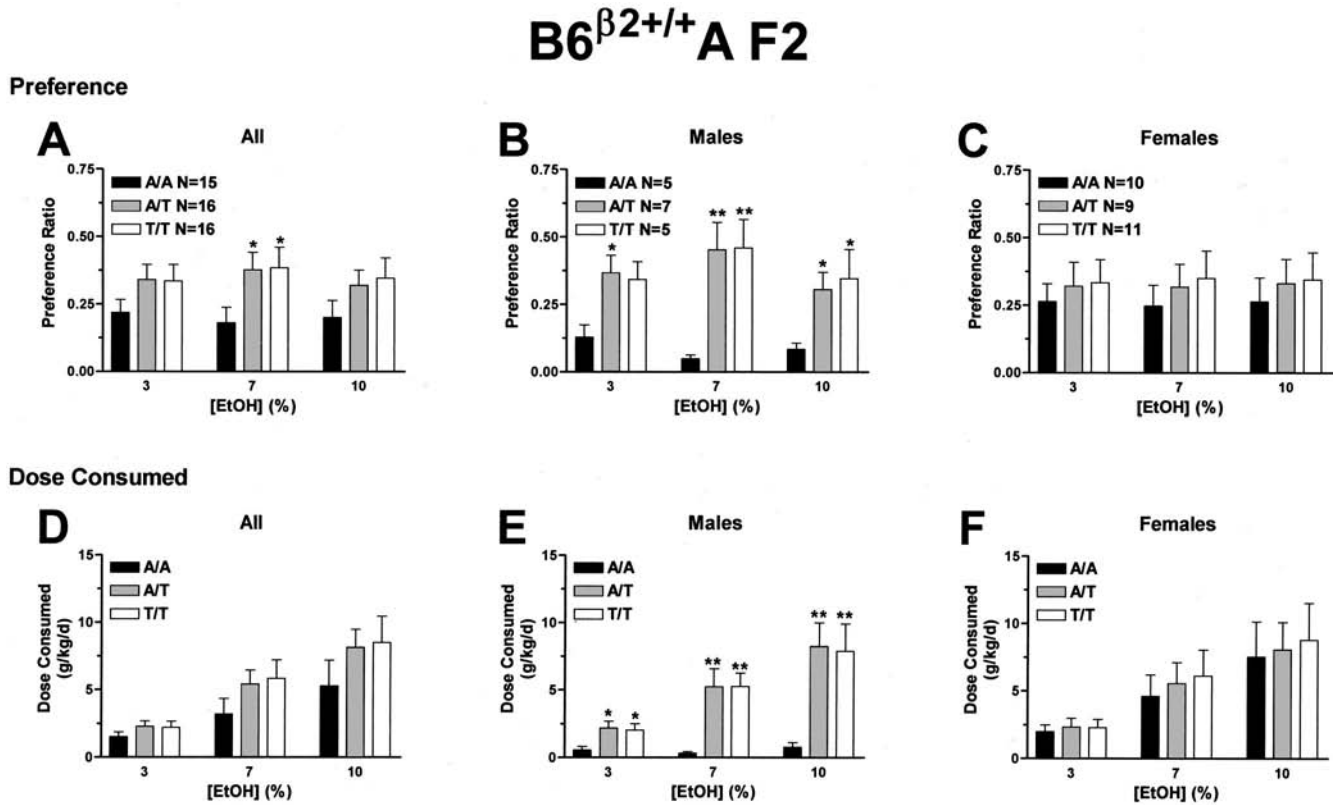


Figure 6. Analysis of *A529T* effects on ethanol (EtOH) preference and consumption in B6 ^{β 2^{+/+}} A F2 mice. Error bars represent standard error of the mean. A: ANOVA (Genotype \times Concentration \times Sex) indicated that an *A529* genotype significantly correlated with decreased ethanol preference. This finding was strongly influenced by the significant effects of genotype in the male data, $F(2, 42) = 22.40, p < .001, (1-\beta) = 1.000$, as determined by a two-way ANOVA (Genotype \times Concentration; B). C: Female mice exhibited the same *A529T* trend, but the effect of genotype was not significant, $F(2, 81) = 1.04, p = .36, (1-\beta) = 0.226$. Similar results were obtained in the consumption data obtained from all mice (D); males (E), $F(2, 42) = 33.70, p < .001, (1-\beta) = 1.000$; and females (F), $F(2, 42) = 0.53, p = .59, (1-\beta) = 0.135$. * $p < .05$ and ** $p < .01$, compared with appropriate A/A data in Bonferroni post hoc test. d = day.

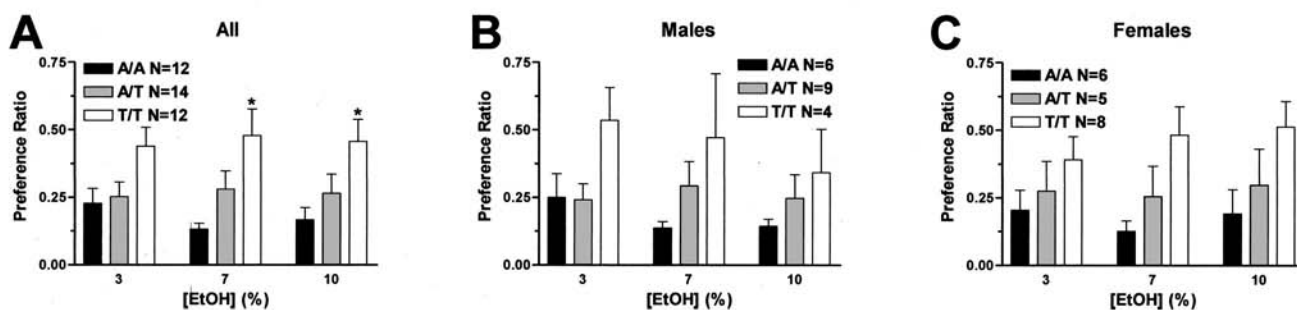
trigeminal ganglion (Gahring, Persiyanov, & Rogers, 2004; Liu, Chang, Jiao, & Simon, 1998) where they may play some role in lingual nociception of nicotine. However, the minimum nicotine concentration to induce this nociceptive response of the tongue is at a nonselective concentration of ~ 60 mM (Carstens, Simons, Dessirier, Carstens, & Jinks, 2000; Wang, Erickson, & Simon, 1993). The maximum nicotine concentrations used in our preference experiments were two orders of magnitude lower. It is therefore most likely that the pharmacological effects, not taste or lingual nociception, of nicotine are responsible for the results we have obtained. The strains with the *A529* genotype appear to be more sensitive to the nAChR activating (Butt et al., 2003; Dobelis et al., 2002) and seizure inducing (Stitzel et al., 2000) effects of L-nicotine than the *T529*-containing strains. These findings suggest that toxic actions of nicotine within the central nervous system may serve to limit intake.

Associations detected in inbred strain analyses may not be due to the candidate gene under investigation but rather due to a gene or genes linked to the candidate gene. Previous work in our laboratory measured nicotine consumption in the heterogeneous

stock (HS) mice that were derived by systematically crossing eight inbred strains. Consistent with the data from the inbred strains, HS mice that were homozygous for the *T529* allele consumed more nicotine than HS mice that were homozygous for the *A529* allele (Tritto et al., 2001). Using F2 mice derived from a cross between the strains C57BL/6J and C3H/HeJ, Li, Karadesh, Jenkins, and Stitzel (in press) also found that mice homozygous for the *T529* allele of *Chrna4* consumed more nicotine than did mice homozygous for the *A529* allele. However, in both of these studies, the association between *Chrna4* genotype and nicotine consumption was not significant. The lack of a significant association between *Chrna4* genotype and nicotine intake in these studies could simply be due to a lack of power. However, these results could be an indication that the relationship between *Chrna4* genotype and nicotine intake is due to a gene linked to *Chrna4*. Therefore, we chose to use the B6 ^{β 2^{-/-}} A F2 intercross in the current study to address the linkage question. The first advantage of these mice is that most, if not all, $\alpha 4$ -containing nAChRs contain $\beta 2$ nAChR subunit, and $\beta 2$ $-/-$ mice do not express the major $\alpha 4$ -containing nAChR that binds nicotine with high affinity ($\alpha 4\beta 2^*$; Marubio et

B6 $\beta^{2-/-}$ -A F2

Preference



Dose Consumed

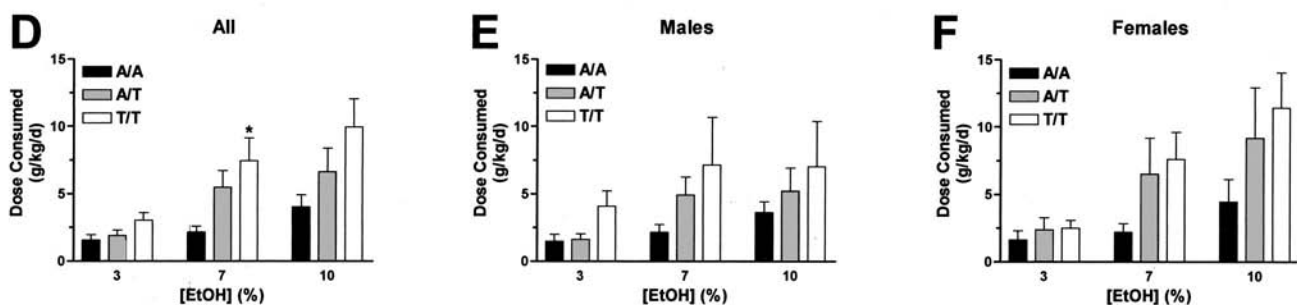


Figure 7. Analysis of *A529T* effects on ethanol (EtOH) preference and consumption in B6 $\beta^{2-/-}$ -A F2 mice. Three-way ANOVA (Genotype \times Concentration \times Sex) detected a significant association of *A529T* genotype with lower ethanol preference in the sex-pooled data (A), $F(2, 96) = 10.10, p < .001, (1-\beta) = 0.983$. Two-way ANOVA (Genotype \times Concentration) showed similar effects of genotype in both the male data (B), $F(2, 48) = 3.62, p = .034, (1-\beta) = 0.642$, and the female data (C), $F(2, 48) = 7.20, p = .002, (1-\beta) = 0.919$, even though the mice lacked *Chrb2*. A significant effect of *A529T* genotype on ethanol consumption was detected in the sex-pooled data (D), $F(2, 96) = 7.42, p = .001, (1-\beta) = 0.935$, and in females (F), $F(2, 48) = 4.74, p = .013, (1-\beta) = 0.776$, but a clear effect was not seen in males (E), $F(2, 48) = 2.98, p = .061, (1-\beta) = 0.552$. No effect of sex was detected in a three-way ANOVA of either the preference or consumption data. Error bars represent standard error of the mean. * $p < .05$, compared with appropriate A/A data in Bonferroni post hoc test. d = day.

al., 1999; Picciotto et al., 1995). Another advantage is that the progenitor C57BL/6 strain carries the *T529* polymorphism, whereas A/J mice carry the *A529* polymorphism (Dobelis et al., 2002). Thus, we were able to determine whether the *Chrna4* polymorphism is associated with nicotine consumption and preference in mice that express $\alpha 4\beta 2^*$ nAChRs and in mice that do not. Significant associations of nicotine consumption and preference with the *A529T* polymorphism were detected in the B6 $\beta^{2+/+}$ -A F2 mice. However, when B6 $\beta^{2-/-}$ -A F2 mice were studied, no reliable associations were found. These findings argue that the *A529T* polymorphism plays a causal role in nicotine intake (i.e., the association is not due to linkage).

We did not find a consistent association between *A529T* genotype and variability in ethanol intake. An effect of the polymorphism on these ethanol-related behaviors was suggested in our initial meta-analysis of data obtained by Belknap et al. (1993) in inbred mouse strains. Similar, but significant, results for ethanol preference were obtained in the meta-analysis of data obtained by Gill et al. (1998) in the AxB/BxA recombinant inbred strains. Gill

et al. (1998) detected a quantitative trait locus for ethanol preference at 107 centimorgans on mouse chromosome 2 using the molecular marker *D2Mit74*. *Chrna4* is found at 108 centimorgans on chromosome 2 (Bessis et al., 1990). Thus, our results with regard to *A529T* genotype (see Table 2) were highly consistent with those obtained by Gill et al. (1998). We also observed an association between *A529T* genotype and ethanol preference/consumption in our B6 $\beta^{2+/+}$ -A F2 mice. The finding that this association was also seen in the B6 $\beta^{2-/-}$ -A F2 mice strongly suggests that a gene (or genes) that is linked to *Chrna4* is responsible for regulating ethanol intake. Meta-analysis of the data obtained by Rodriguez et al. (1994, 1995) in the BxD recombinant inbred strains also point to a linkage effect. Although there is a trend, there is no significant association of *A529T/D2Mit200* genotype with ethanol consumption in the BxD recombinant inbred strains. In contrast, a significant effect of *A529T* genotype on ethanol consumption in HS mice (Tritto et al., 2001) and in an F2 intercross between C57BL/6J and C3H/HeJ mice (Li et al., in press) has been reported. However, in both studies, the consumption

trend is in the opposite direction; *A529* mice consumed more ethanol than *T529* mice. Therefore, it is highly likely that genes other than *Chrna4* are responsible for regulating this phenotype.

It is possible that other nicotinic subunits or genes linked to the null mutation of *Chrb2* could provide some functional compensation for the loss of *Chrb2*. However, the evidence for functional compensation causing a confound in our current results is sparse. The $\alpha 4\beta 2^*$ nAChR is the predominant nicotinic subtype in mammalian brain (Guo & Chiappinelli, 2002; Role & Berg, 1996), and null mutation of *Chrb2* destroys the high-affinity nicotine binding site (Picciotto et al., 1995). Deletion of *Chrb2* does not appear to alter the expression of other nAChR subunits (Picciotto et al., 1995). The nAChRs remaining after *Chrb2* deletion are believed to be $\alpha 3\beta 4^*$ and $\alpha 7$ (Marks et al., 2002; Picciotto et al., 1995; Whiteaker et al., 2000). Thus, $\beta 4^*$ and $\alpha 7$ nAChRs probably mediate some responses to nicotine and possibly alcohol in animals lacking *Chrb2*. Indeed, $\beta 2^{-/-}$ animals exhibit hypothermic responses to nicotine, but the dose–response curve of the mutants in nicotine-induced hypothermia is shifted significantly to the right in comparison to wildtype animals (Tritto et al., 2004). This finding is consistent with the lower affinity of $\beta 4$ -containing nAChRs for nicotine in radioligand binding assays (Parker, Beck, & Luetje, 1998; Parker, Harvey, & Luetje, 2001).

In conclusion, the *A529T* polymorphism in *Chrna4* plays a pivotal role in nicotine consumption and preference. This effect most likely occurs through a change in the sensitivity of the $\alpha 4$ -containing nAChR to nicotine that is conferred through the polymorphism (Butt et al., 2003; Dobelis et al., 2002). Although the polymorphism alters the sensitivity of the $\alpha 4\beta 2^*$ nAChR to ethanol (Butt et al., 2003) and is important in modulating variability in ethanol withdrawal (Butt et al., 2004), its role in ethanol intake is inconsistent and most likely due to linkage with a nearby gene or genes on chromosome 2.

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