Transgenic Over Expression of Nicotinic Receptor Alpha 5, Alpha 3, and Beta 4 Subunit Genes Reduces Ethanol Intake in Mice

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Abstract

Abuse of alcohol and smoking are extensively co-morbid. Some studies suggest partial commonality of action of alcohol and nicotine mediated through nicotinic acetylcholine receptors (nAChRs). We tested mice with transgenic over expression of the alpha 5, alpha 3, beta 4 receptor subunit genes, which lie in a cluster on human chromosome 15, that were previously shown to have increased nicotine self-administration, for several responses to ethanol. Transgenic and wild-type mice did not differ in sensitivity to several acute behavioral responses to ethanol. However, transgenic mice drank less ethanol than wild-type in a two-bottle (ethanol vs. water) preference test. These results suggest a complex role for this receptor subunit gene cluster in the modulation of ethanol’s as well as nicotine’s effects.

Keywords

Nicotine; ethanol; transgenic mice; nAChR subunits; preference drinking; dependence

Introduction

Studies in cell lines have demonstrated that nicotine and alcohol (ethanol) interact at nicotinic acetylcholine receptors (nAChRs), altering their expression levels (Dohrman and Reiter, 2003) and modulating their agonist response (Marszalec et al., 1999). nAChRs are
allosteric membrane proteins that belong to a large family of ligand-gated ion channels composed of combinations of five subunits (alpha 2 - alpha 10, beta 2 - beta 4) forming channel-receptor complexes with varied functional and pharmacological characteristics that are specified by their composition.

Some studies have used lines of mice (Tritto et al., 2001; Tritto et al., 2002) and rats (de Fiebre et al., 2002) selectively bred to be sensitive or resistant to the high-dose (anesthetic) effects of ethanol. By analyzing the responses of these selected lines as well as a number of recombinant inbred strains developed from their crosses genotype-specific responses to alcohol have been shown that suggest the role of alpha 4 and alpha 6 nAChR subunits in modulating ethanol sensitivity. Other studies with nAChR mutant mice (Butt et al., 2003; Owens et al., 2003) support a common physiological basis for alcohol and nicotine’s behavioral effects. Alcohol and tobacco, specifically nicotine, are often co-abused substances. The contributions of various nAChR subunits to behavioral responses to nicotine and nicotine dependence in mice have recently been reviewed (Changeux, 2010). Recent genetic association studies have implicated sequence variants in the CHRNA5/A3/B4 gene cluster, located on human chromosome 15q25, in alcohol use disorders (Joslyn et al., 2008; Schlaepfer et al., 2008a; Wang et al., 2009) and nicotine dependence (Agrawal et al., 2008; Baker et al., 2009; Berrettini et al., 2008; Chen et al., 2009; Gruca et al., 2010; Saccone et al., 2009; Spitz et al., 2008; Stevens et al., 2008; Thorgerisson et al., 2008; and see Beirut, 2010; Schlaepfer et al., 2008b for review). However, little is known about the functional impact of these polymorphisms on nicotinic receptor function or their effects on the development of a brain susceptible to nicotine and alcohol abuse or dependence. Different authors have suggested that the effects of different polymorphisms on risk for co-morbid abuse of the two drugs may be different, mediated by altering the pharmacological responses or by modifying the expression level of nAChRs (Wang et al., 2009; Falvella et al., 2010). Knockout mouse lines exist for the alpha 5 and beta 4 subunit genes, but the alpha 3 subunit knockout is lethal. Null mutants for alpha 5 and beta 4 genes showed reduced somatic withdrawal from chronic nicotine precipitated by mecamylamine injection (reviewed in Changeux, 2010). Moreover, deletion of the alpha 5 subunit subunit has been recently associated with an enhanced nicotine self-administration (Fowler et al., 2011), while mice overexpressing the beta 4 subunit showed aversive effects to nicotine (Frahm et al., 2011).

In addition, recent pharmacological studies have implicated α3β4 nAChR in ethanol consumption and seeking in rats (Chatterjee et al., 2011) as well as in alcohol and nicotine co-dependencies (Bito-Onon et al., 2011). Also, the non-specific nAChR antagonist mecamylamine attenuates ethanol-induced stimulation in DBA/2J mice (Kamens and Phillips, 2008). Although mecamylamine is regarded as non-specific, low doses of this nAChR antagonist have been shown to be more specific for α3β4 nAChR (Papke et al., 2001). Furthermore, α3-containing nAChRs have been involved in acute ethanol effects since studies of Jerlhag et al., (2006) and Larsson et al., (2004) using α-conotoxin MII (α3β2, β3 and α6 specific), but not α-conotoxin PIA analogue (α6 specific) showed attenuated ethanol-induced locomotor stimulation. These findings are also supported by the work of Kamens et al., (2009) showing that mice that were less sensitive to ethanol-induced locomotor stimulation overexpressed alpha 3 subunit in whole brain.

To elucidate further the potential role of the CHRNA5/A3/B4 gene cluster in modulating sensitivity to ethanol in mice, we have generated a BAC transgenic (TgCHRNAS5/A3/B4) model (Gallego et al., in revision). Our previous studies with these mice strongly demonstrate the general involvement of these subunits in nicotine’s reinforcing effects, as TgCHRNAS5/A3/B4 (Tg) mice show greater nicotine self-administration and enhanced motivation to obtain nicotine (higher break point in a progressive ratio schedule) than wild-type (WT) littermates. These animals also show enhanced responses to acute nicotine...
(greater reductions in activity and enhanced sensitivity to nicotine-induced seizures) as compared with WT, along with increased [125I]-epibatidine binding in olfactory bulb, CA1 region of hippocampus, superficial gray area of the superior colliculus and pyriform cortex (Gallego et al., in press) and increased total $^3$H nicotine binding (Molas et al., 2010, Viñals et al., in press).

We therefore studied alcohol drinking in Tg vs. WT mice using a standard two-bottle preference test. We also studied their preference for a sweet and bitter solution, as well as their sensitivity to several acute effects of ethanol. Unexpectedly, our results demonstrate that transgenic mice showed reduced ethanol intake, opposite to their greater self-administration of nicotine, without modifications in any acute ethanol responses we tested.

**Methods**

**Animals**

Tg$CHRNA5/A3/B4$ mice over expressing the human nicotinic gene cluster containing the alpha 5, alpha 3 and beta 4 subunits and B6SJLF1/J WT are maintained in our colonies at the animal facility of the PRBB (Biomedical Research Scientific Park, Centre for Genomic Regulation, Barcelona, Spain). Transgenic mice were obtained in heterozygosity on a B6SJLF1/J genetic background by standard pronucleus microinjection of the 111 kb BAC fragment inserted with the human cluster containing the three nicotinic receptor subunits (alpha 3, alpha 5, and beta 4) on B6SJLF1/J genetic background, as previously described (Gallego et al. 2011, in press). The presence of all promoter regions was assessed by PCR on maxiprep-extracted DNA from the RP11-335K5 (AC067863) clone, and rearrangements within the BAC were checked (EagI (BshTI) restriction pattern). Two lines were generated carrying between 16–18 (Line 22 Tg) and 4–5 copies (Line 30 Tg) of the transgene, respectively, as detected by Slot-blot (data not shown). No significant differences between genotypes were found in endogenous alpha 3, alpha 5, and beta 4 subunits expression in the cerebral cortex when endogenous $\text{Chr}a3$, $\text{Chr}a5$ and $\text{Chrn}b4$ were checked by qRT-PCR analysis. Western blot analysis confirmed increased expression of beta 4 and alpha 3 subunits in specific regions of transgenic mice in both transgenic lines compared to WT (Gallego et al. 2011, in revision). Hybrid founders were crossed using B6SJLF1/J females and all experiments were performed using mice from the F1–F5 generation to attenuate littermate’s genetic differences. The non-transgenic littermates obtained from crosses of males Tg$CHRNA3/A5/B4$ mice and B6SJLF1/J females served as controls. Even though in the molecular characterization of the lines we could not detect major differences, we decided to use two lines [L22 and L30] and examine data from each separately to discount any possible positional effects of the transgene.

Where no differences were detected the data are merged for the two lines. Male mice weighing 30 to 35 g (8–12 weeks old) at the beginning of the experiments were used in this study. Naïve mice were housed individually in a temperature (21° ± 1°C), humidity (55% ± 10%), and light-cycle controlled room (lights on between 08:00 and 20:00). Food and water were available ad libitum except during the exposure to the behavioral paradigms. Mice were handled daily for 1 week before starting the experiments. Studies were conducted with two batches of mice (see Behavioral testing). All experimental procedures were approved by the local ethical committee (CEEA-PRBB), and met the guidelines of the local (Catalan law 5/1995 and Decrees 214/97, 32/2007) and European regulations (EU directives 86/609 and 2001-486) and the Standards for Use of Laboratory Animals nº A5388-01 (NIH). The CRG is authorized to work with genetically modified organisms (A/ES/05/I-13 and A/ES/05/14). Experiments were performed under conditions blind to genotype and treatment group.
Binding assays

Membrane preparations from cortex and hippocampus of WT (n = 12) and TgCHRNA3/A5/B4 (n = 12) were obtained as previously described (Davies AR et al., 1999). Saturable binding of [3H] nicotine (Anderson et al., 1994) (81 Ci/mmol, Amersham Biosciences, UK) or the competitive antagonist selective for α7-homopentamers [3H] methyllycaconitine (MLA, 38.39 Ci/mmol, American Radiochemical, USA) to P2 membranes was determined by incubating a single concentration of radioligand. P2 samples were incubated with 10 nM of [3H] nicotine, which gives the best ratio of specific to non-specific binding, and Tris-HEPES buffer, pH 7.4, 30 min at room-temperature and 1 h at 4°C. Nonspecific binding was determined by 100 μM (−) nicotine. The [3H] MLA assay was carried out as described (Sharples CG et al., 2000). P2 samples were incubated with 2 nM of [3H] MLA in 50 mM phosphate buffer, pH 7.4, supplemented with 0.1% (w/v) BSA during 2.5 h at room temperature, nonspecific binding determined by 1 μM unlabeled MLA. After incubation, samples were filtered under vacuum with a Brandel Cell Harvester (Semat Technical, UK). Filters were placed in scintillation vials containing 2.5 mL of Ready SafeGTM scintillation liquid (PerkinElmer, USA) and counted using a Wallac 1409/1411 liquid scintillation spectrometer (Pharmacia, Finland) at an efficiency of 19–21%.

Drugs

Ethyl alcohol was purchased from Merck Chemicals (Darmstadt, Germany) and diluted in tap water for the preference drinking studies or in physiological saline (0.9% NaCl) when injected intraperitoneally (i.p.). Solutions were made fresh daily. Ethanol doses were i.p. injected according to body weight in a volume of 0.1 mL/10 g. Saccharin (Merck Chemicals, Darmstadt, Germany) was diluted in tap water to 0.033%. Quinine HCl (Sigma-Aldrich, MO, USA) was diluted in tap water to 0.1 mM (Batch 1 mice) or 0.2 mM (Batch 2 mice).

Behavioral testing

Specific testing protocols are given in subsequent sections. Mice were tested in two batches, each including mice of both transgenic lines and wild-types. Batch 1 comprised 20 Tg (10 L22, 10 L30) and 18 WT mice (10 L22, and 8 L30). Batch 2 comprised 20 Tg (11 L22, 9 L30), and 22 WT mice (10 L22, and 12 L30). Most data reported correspond to Batch 1 mice. We first tested Batch 1 mice for two-bottle ethanol preference drinking, followed by saccharin and quinine preference. After 34 days, mice were tested for performance on the balance beam. Eight days later, these mice were tested for ethanol stimulated locomotor activity. Starting 7 days later, mice were tested for ethanol hypothermia and for performance on the accelerating rotarod. One week later, all mice were tested for ethanol-induced loss of righting reflex and acute ethanol withdrawal severity. Mice in the second batch were tested only for ethanol preference drinking at the 10% and 20% concentrations, followed by quinine preference drinking, using a higher quinine dose.

Ethanol preference drinking—Voluntary ethanol consumption and preference were examined using a variant of our usual two-bottle free-choice paradigm (Crabbe et al., 1996). All mice had 24 h access to two inverted 25 mL tubes with metal sippers placed on stainless steel cage tops. Food was distributed near both bottles to avoid food associated tube preferences. One tube always contained tap water and the other contained increasing concentration of ethanol (3%, 6%, 10%, 20% vol/vol in tap water). Mice had access to each concentration for 4 days with bottle positions alternated every two days. Fresh fluids were provided each time the concentration was changed. Each morning, daily fluid intake was recorded from both bottles by measuring the level of the meniscus on the graduated drinking tube. Ethanol 3%, 6%, 10%, 20% and water tubes placed on two empty cages allowed for the measurement of leakage and evaporation from the tubes. The average volume depleted
from these “control” tubes was subtracted from the individual drinking volumes each day before data analysis. To obtain a measure of ethanol consumption that corrected for individual differences in mouse size, g of ethanol consumed per kg of body weight per day were calculated for each mouse. Average ethanol intake per day was calculated for each ethanol concentration. The ethanol preference ratio was calculated daily as volume of ethanol solution consumed per total volume of fluid (water plus ethanol solution) consumed. Body weight was recorded simultaneously with each solution change, and cages were changed every 8 days in the middle of each ethanol block to avoid interaction with changes in ethanol concentrations. Batch 2 mice were started at 10% ethanol vs. water for 4 days and then 20% ethanol for 4 more days.

**Saccharin and Quinine Consumption**—After finishing voluntary ethanol consumption, mice in Batch 1 were given two tubes containing water for 2 days and then were given plain water in one tube and saccharin (0.033% solution) in the other pipette, using a protocol similar to that described for ethanol consumption. After 4 days exposure to the saccharin solution, it was replaced by a quinine solution (0.1 mM) and mice had 4 days of access to quinine vs. water. Consumption (mg/kg) and preference for each solution were calculated as described for ethanol. As noted, mice in Batch 2 had 8 days of ethanol preference testing, then 2 days of water only, and were then tested for quinine preference at a higher dose (0.2mM) for 4 days.

**Balance beam test**—This apparatus and test have been described elsewhere (C rabbe et al., 2003; Escorihuela et al., 1995; Escorihuela et al., 1998). The flat-surfaced balance beam (20 mm wide × 104 cm long) was a piece of pine molding. The beam was elevated 54.5 cm above the table top and pads were placed under the beam to provide cushioning if an animal fell. On the day before data collection, subjects were trained on the beam. Each mouse was allowed to traverse the beam once and was then returned to its home cage. Most mice simply run across the beam in a few seconds. However, if a mouse would not cross the beam it was nudged gently or its tail was touched to re-initiate movement. After all mice were trained, they were returned to the colony room. As mice made very few errors during this drug-free training with no observable genotype-dependent differences, these data were not recorded. Twenty-four hours later, the mice were again moved to the test room and habituated for 1 h. Each subject was injected with ethanol (1.2 g/kg) and placed back in its home cage for 10 min. Each mouse was then tested for a single crossing on the beam. Hind footslips were recorded.

**Locomotor activity**—Locomotor responses induced by an acute injection of ethanol (2.0 g/kg) were evaluated using activity boxes (9 × 20 × 11cm, Imetronic, Lyon, France). The boxes were provided with two lines of 14 photocells each, one 2 cm above the floor to measure horizontal activity, and the other located 6 cm above the floor to measure vertical activity (rears), in a low luminosity environment (5 lux). Mice were placed in the activity cages for a period of 20 min and baseline activity was recorded for the last 10 min. After that, mice were returned to their home cages and injected with ethanol (2.0 g/kg). Five minutes after injection, animals were replaced in the monitors and their locomotor activity was recorded during 10 min. Activity was analyzed as number of lower and upper beam breaks in order to study horizontal and vertical activity.

**Accelerating rotarod test (ARR)**—Ataxia in response to an acute injection of ethanol (2.0 g/kg) was evaluated using the accelerating rotarod paradigm with 4 to 40 revolutions per minute (rpm) over 2 min (five-line accelerating rotarod; LE 8200, Panlab, Barcelona, Spain). Three days before ethanol data collection, mice were trained on the rotarod for 10 consecutive trials with an inter-trial interval of 30 s. On the test day, mice were given 3
consecutive rotarod trials: average rpm at the time the mouse fell was recorded as a baseline measure. Mice were immediately injected with a single dose of ethanol (2.0 g/kg). Giving 3 additional rotarod trials 30 min after injection assessed ethanol impairment on the rotarod.

**Hypothermia (HT)—**Hypothermic response to an acute injection of ethanol (2.0 g/kg) was evaluated by manually restraining the mouse, inserting a thermo-coupled flexible probe about 2 cm into the rectum for 10 s, and recording temperature using a digital thermometer (Panlab, Barcelona, Spain). On the test day, mice had a baseline temperature taken and were immediately injected with a single dose of ethanol (2.0 g/kg). Temperatures were recorded 45, 90 and 135 min after injection.

**Ethanol-induced loss of righting reflex (LORR) and acute ethanol withdrawal severity—**Details of the procedure for the assessment of sensitivity to the loss of righting reflex after ethanol have been discussed elsewhere (Crabbe et al., 2006a). Briefly, mice were injected with 4.0 g/kg ethanol, which causes the animals to lose righting reflex within 2–3 min. Each mouse was then placed on its back on the bedding in its home cage and was considered to display LORR if it was no longer able to right itself within 30 s. Mice were then observed until they first righted themselves, at which point they were immediately turned on their back again. If they righted themselves for a second time within 30 s, the time after injection was noted; if not, they were returned to the experiment until they regained the righting reflex.

Acute EtOH withdrawal severity was measured in the same mice at the same time using as an index the handling-induced convulsion (HIC). Before injection, mice were scored for the HIC twice, twenty min apart, and the average HIC score was taken as their baseline. Mice were then injected with 4.0 g/kg ethanol and scored for duration of LORR as described above. Nearly all mice had recovered their righting reflex within 2 h, and only 2 mice still showed LORR at 4 h after injection. Starting 2 h after injection, each mouse was picked up by the tail and observed for HIC signs. If no signs were elicited, the mouse was spun gently by the tail through a \(180^\circ–360^\circ\) arc and again observed. HIC scores range from 0 to 7 and vary from myoclonus through severe tonic-clonic convulsions elicited simply lifting the mouse by the tail (score = 6; a score of 7 is a lethal tonic hindlimb extensor seizure and is not seen after acute injection). The details of this procedure and its scoring have been published (Crabbe et al., 1991; Metten and Crabbe, 2005). Mice were scored hourly for the HIC between hours 2 and 12 post-injection. Area under the HIC time-course curve (AUC), and peak HIC score were calculated; peak score is taken as the average of the three greatest HIC scores occurring in succession.

**Statistical analyses**

In the initial analysis of all experiments, multifactor ANOVAs were used with genotype (WT vs. Tg) as one factor and line (L22 vs. L30) as another, in order to ascertain whether there were any line-specific effects. Although there were occasional main effects of line, we saw no significant interactions of line \(\times\) genotype in any analysis except for locomotor activity. Therefore, data except those for activity were combined across the L22 and L30 Tg (and, similarly, their WT) lines and we reanalyzed all data ignoring line. Drinking data were analyzed according to our usual practice for such studies (Crabbe et al., 1996). Differences in drinking between genotypes were analyzed using the g/kg/day consumed as the primary dependent measure for ethanol or mg/kg/day for other tastants. Other dependent measures were the preference ratio (calculated as the volume of ethanol, saccharin, or quinine consumed divided by the total volume consumed) and the total fluid consumption (mL/g body weight). Intakes (g/kg or mL/g) and preference ratios were averaged for the 4 days each concentration was offered. ANOVAs then initially assessed Genotype and
Concentration (repeated measure) effects on the dependent variables. Separate analyses were performed for each tastant (saccharine or quinine) using ANOVA for comparisons between genotypes.

Effects of ethanol on locomotor activity were tested by using analysis of variance on genotype and line with the drug effect assessed as a repeated measure, followed by post hoc analysis for individual differences when required. Data from balance beam and the LORR tests were analyzed using ANOVA to compare genotypes. For the ten ARR training trials, we assessed genotype and trial (repeated measure) effects. A similar analysis was used for baseline and post-ethanol HT scores. For the ethanol test on ARR, ANOVA on the pre- vs. post-ethanol rpm at time of fall as a repeated measure was compared across genotypes. For withdrawal severity, area under the HIC time course curve (AUC) and peak HIC score (average of the 3 highest consecutive HIC scores) were analyzed using ANOVA for comparisons between genotypes.

In all experiments differences were considered significant if P < 0.05. The SPSS statistical package was used.

Results

Binding assays

Analysis of $^3$H nicotine binding showed an increase of two to three fold in transgenic hippocampal membranes, as compared to WT (WT = 9.62 ± 3.16 fmol/mg protein; Tg = 27.42 ± 2.88 fmol/mg protein; P < 0.05). In cortical preparations we also observed an increase of nicotine binding sites (WT = 12.86 ± 1.39 fmol/mg protein; Tg = 18.96 ± 2.03 fmol/mg protein; P < 0.05). No significant differences were detected in $^3$H MLA binding.

Ethanol preference drinking

Alcohol consumption was measured in WT and Tg mice using the two-bottle choice paradigm as described above. Testing was conducted over a 16-day period. Figs 1A and 1B show the average daily consumption and preference ratios, respectively. Tg mice drank less than WT. Analyses of the consumption data showed significant effects of Genotype [F(1, 35) = 4.65, P = 0.038] and Concentration of EtOH [F(3, 33) = 25.43, P < 0.001]. The interaction of Concentration and Genotype was not significant [F(3, 33) = 1.25, n.s.]. For the preference data, there were also significant effects of Concentration of EtOH [F(3, 33) = 37.78, P < 0.001], but neither the effect of Genotype [F(1,35) = 2.15, n.s.] nor the Day × Genotype interaction were significant [F(1,33) = 0.20, ns].

Alcohol drinking data from the second batch (not shown) resembled those from the first batch. There was a significant effect of Genotype [F(1,34) = 6.08, P = 0.019]; however, the second batch of mice showed no significant effects of Concentration of EtOH [F(1, 34) = 0.34, n.s.]. As in the first batch, the interaction of Day and Genotype was not significant [F(1,34) = 0.49, n.s.]. For the preference data, there was a tendency for Tg mice to show lower preference ratios than WT, but the effect of Genotype [F(1,34) = 3.34, P = 0.077] was only a trend. There was a significant effect of Concentration of EtOH [F(1, 34) = 100.58, P < 0.001] but again the Day × Genotype interaction was not significant [F(1,34) = 0.85, n.s.]. No significant differences between genotypes were found in the total fluid consumed by Batch 1 mice [F(1, 35) = 0.04, n.s., Fig. 1C] or Batch2 [F(1, 34) = 0.92, n.s.].

Saccharin and quinine consumption were used as taste controls (Fig. 1D). In Batch 1, there were no differences between genotypes in consumption of [F(1, 35) = 0.00, n.s., Fig. 1D] or preference for saccharin [F(1, 35) = 0.14, n.s., Fig. 1E]. Both genotypes clearly preferred saccharin as evidenced by the preference ratios. For quinine, genotypes also did not differ in
consumption \[F(1, 35) = 2.14, \text{n.s., Fig. 1D}\], or preference \[F(1, 35) = 0.07, \text{n.s., Fig. 1E}\]. However, we noticed that the quinine preference scores for both genotypes were close to 50%. Thus, it was impossible to tell whether or not the mice could taste the quinine. Therefore, in the second batch of mice, we tested for quinine preference using a higher concentration (0.2 mM). Again, genotypes did not differ, showing no avoidance of quinine (data not shown).

**Balance beam**

The number of foot slips in the balance beam test was analyzed after EtOH injection (Fig. 2). There were no significant differences in the number of foot slips between WT and Tg mice \[F(1, 33) = 0.52, \text{n.s.}\]. Because mice make virtually no foot slips when not intoxicated (Crabbe et al., 2003), this suggests a similar response to EtOH in both genotypes.

**Locomotor activity**

Activity counts measured during the 10 min test are presented in Fig. 3. Since in this test we tended to find significant interactions between genotype and line, both in horizontal \[F(1, 31) = 4.14, P = 0.05\] and vertical activity \[F(1, 31) = 3.99, P = 0.05\], we report the subsequent statistical analysis separated by lines. Ethanol reduced horizontal activity in mice of both L22 \[F(1, 17) = 132.43, P < 0.001\] and L30 \[F(1, 14) = 28.88, P < 0.001, \text{Fig. 3A}\]. However we found a significant interaction between genotype and treatment only in mice of L22 \[F(1, 17) = 7.21, P < 0.05\]. This was due to greater baseline activity in Tg L22 mice than in WT \[F(1, 17) = 4.34, P = 0.05\] without a significant difference between genotypes after ethanol injection \[F(1, 17) = 1.97, \text{n.s.}\]. The effects of ethanol (and ethanol × genotype) on vertical activity paralleled those on horizontal activity, and yielded equivalent statistical outcomes (F values not shown). However, note that vertical activity was nearly completely suppressed by ethanol in all genotypes.

**Accelerating rotarod (ARR)**

Data from the training and the test session are shown as rpm reached before falling in Fig. 4. Mice of both genotypes improved across the 10 training sessions \[F(9, 26) = 6.84, P < 0.001, \text{Fig. 4A}\]. There was no significant difference between WT and Tg mice either on average \[F(1, 34) = 1.93, \text{n.s.}\] or across the training session \[\text{Genotype × Trial interaction, } F(9, 26) = 0.87, \text{n.s.}\].

When mice were subsequently tested before and after ethanol injection, both genotypes showed significant impairment after injection \[F(1, 34) = 39.86, P < 0.001\]. Tg mice performed worse than WT overall \[F(1, 34) = 7.62, P < 0.01\], but the interaction between genotype and treatment was not significant \[F(1, 34) = 0.19, \text{n.s., Fig. 4B}\].

**Body temperature**

Data from body temperature are shown in Fig. 5. A separate analysis of baseline temperature showed no significant differences between genotypes \[F(1, 34) = 0.22, \text{n.s.}\]. Ethanol reduced body temperature significantly \[F(3, 32) = 60.53, P < 0.001\]. However, there was no significant difference between WT and Tg \text{CHRNA5/A3/B4} mice over time \[F(3, 32) = 0.31, \text{n.s.}\]

**Ethanol-induced loss of righting reflex (LORR)**

Sensitivity to sedative effects of ethanol was measured by the duration of LORR (Fig. 6). Of the total of 19 Tg and 14 WT mice tested, 5 Tg and 4 WT mice did not lose righting reflex. This could have been because the injection missed the intraperitoneal cavity or alternatively, they could have been insensitive to ethanol. We conservatively decided to eliminate them...
from the data set for analysis. As LORR is a threshold, all-or-none response, its magnitude is usually quantified by its duration. The analysis of the remaining 14 Tg and 10 WT mice showed no significant differences between genotypes in the duration of LORR \(F(1, 20) = 0.43, \text{n.s.}\). An additional analysis of LORR that included all the animals that failed to lose righting reflex by giving them scores of zero duration yielded a nearly identical outcome (data not shown).

**Acute ethanol withdrawal severity**

Ethanol withdrawal severity was measured in the same mice tested for LORR by HICs after an acute injection of EtOH (4.0 g/kg); the acute withdrawal time course is shown in Fig. 7A. Baseline HIC scores were first analyzed as the average of the two pre-injection data points. No significant differences were found between genotypes in baseline HIC score \(F(1, 29) = 0.08, \text{n.s.}\). To summarize the withdrawal severity, we computed the area under the time-response curve (AUC) without correcting for the very low baseline scores. We also computed the peak score (Fig. 7B, C). Neither AUC nor peak withdrawal scores differed significantly between WT and transgenic mice [both \(F(1, 29) < 1\), Fig. 7A, B, C].

**Discussion**

Alcohol and nicotine addiction are often treated as separate disorders, although ~60–80% of heavy drinkers smoke tobacco (Moss et al., 2007), and it has been suggested that common genes are involved in the susceptibility to both alcohol and nicotine dependence. Recent human genetic association studies have identified a genetic locus, encoding for the alpha 3 \((CHRNA3)\), alpha 5 \((CHRNA5)\), and beta 4 \((CHRNBS4)\) nAChR subunits, in nicotine and alcohol-dependent subjects (Joslyn et al., 2008; Saccone et al., 2009; Wang et al., 2009), suggesting an involvement of these specific subunits in alcohol dependence. We have here studied alcohol drinking in transgenic mice overexpressing the human cluster. Our results demonstrate that transgenic mice consume less ethanol than wild types, a pattern opposite to their greater self-administration of nicotine. We found that overexpressing animals did not differ in several other responses to acute ethanol.

In our experiments, the lower consumption by Tg mice than WT of alcohol solutions did not appear to be due to any difference in their sensitivity to detect or their preference for sweet taste, as the genotypes did not differ in preference for a highly preferred concentration of saccharin. However, we were unable to completely rule out the potential role of avoidance of bitter taste as neither of the two concentrations of quinine tested was avoided by Tg or WT mice. It is possible that we employed concentrations that were too low to be detected by this strain, although prior studies with many null mutants have used concentrations in this range and reported avoidance in WT (usually C57BL/6J) and mutants (Crabbe et al., 2006b). Furthermore, a concentration of 0.8 mM is normally avoided by several inbred mouse strains including C57BL/6J and SJL/J (Lush, 1984). Alexander Bachmanov and colleagues (personal communication) showed that C57BL/6J and (to a somewhat lesser extent) SJL/J mice strongly avoided quinine concentrations greater than or equal to 0.1 mM. Thus, it seems unlikely that the mice could not detect the solutions, but we do not know why they did not avoid drinking them. It is possible that the fact that we tested for quinine preference after both ethanol and, for batch 1 mice, saccharin preference resulted in a carry-over effect on the quinine taste test, as we have recently reported that prior alcohol preference tests can influence results with subsequent taste preference tests (Crabbe et al., 2011). We consider the control for sweet taste to be more critical, given the substantial evidence for some common genetic control of preference for sweet flavors and alcohol in both laboratory animals and humans (see Kampov-Polevoy et al., 1999).
The drinking differences between Tg and WT mice are not large. During the 4 days of access to 10% ethanol vs water, the Tg mice whose data are shown in Figure 1A drank 4.77 ± 0.95 g/kg day vs 6.25 ± 0.82 for the WT. This represents intake reduced 23.6% from WT amounts. Differences of this magnitude, however, are as large as those often reported for gene-targeted mice when tested for ethanol preference drinking (Crabbe et al, 2006b). They are repeatable, as both Batch 1 and Batch 2 Tg mice drank less than WT despite the differences in the series of concentrations offered. The differences are also robust statistically, as significant differences were seen whether analyzing the data day by day or summarized across concentrations, as we report here. The differences in intake were not accompanied by reliable reductions in preference ratios. Figure 1B shows the expected inverted-U relationship between ethanol preference ratio and concentration. It also shows that both genotypes of mice displayed systematic increases in preference during the 3rd and 4th days at each concentration as compared with Days 1 and 2. This could have resulted from side preferences, as the position of the ethanol bottle was changed after each 2 days; alternatively, the mice could be displaying neophobia for each new ethanol concentration when initially offered. Whichever is the case, the patterns were identical in Tg and WT, so this environmental effect could not explain the systematic differences in intake.

Intake is preferred over preference ratio to index ethanol’s reinforcing efficacy. However, we do not know whether the reduced drinking represents reduced sensitivity to ethanol’s rewarding effects. We previously showed that TgCHRNA5/A3/B4 has increased expression of the β4, α3 and α5 subunits (Gallego et al., in revision) and here demonstrate increased nicotine binding. Moreover, we also observed reduced activation of VTA dopaminergic neurons upon nicotine injection, which may reflect alterations in the aversive properties of nicotine and/or ethanol in our transgenic mice (Gallego et al., in revision). Testing for an ethanol conditioned place preference or an ethanol conditioned taste aversion could help to evaluate this inference (Cunningham and Phillips, 2003). Results of a complex analysis of microinjection of nicotine and ethanol into specific brain regions suggest that activation of some nicotinic receptors may potentiate ethanol’s efficacy to support a conditioned place preference in Wistar rats (Zarrindast et al., 2010). Nonetheless, a review of many genetic studies across mice and rats concluded that two-bottle preference drinking appears to be a reasonable assessment of ethanol’s rewarding effects in most cases (Green and Grahame, 2008). Neither do we know whether motivation for ethanol was different - testing these animals for oral operant self-administration on a progressive ratio schedule could establish whether this is the case. An alternative might be different rates of ethanol metabolism between Tg and WT. For example, Syrian golden hamsters drink very large amounts of ethanol in preference tests, but they also have a high rate of ethanol elimination, and do not reach meaningful blood ethanol concentrations (Green et al., 2004). We believe the most likely interpretation of these data is that the nicotinic gene cluster alters either ethanol’s reward value or motivation to obtain the drug.

Although we have not measured BECs after drinking, it is not very likely that the amounts ingested by either Tg or WT mice would yield intoxicating blood ethanol concentrations since both genotypes consume low levels of ethanol after 24h of voluntary ethanol consumption as compared with data from many mouse genotypes (Wahlsten et al., 2006). Moreover, there is ample evidence in the literature that 24h of voluntary alcohol consumption does not yield BECs that have a measurable intoxicating effect even in C57BL/6 mice, a high alcohol-prefering strain (see, for example Dole and Gentry, 1984). There are also data from other models genetically predisposed to drink ethanol where the animals limit their voluntary drinking to amounts that can be readily metabolized, possibly in an attempt to avoid reaching intoxication (Gill et al., 1986). Many of these studies were reviewed in (Rhodes et al., 2005) where we introduced another model of alcohol consumption, the Drinking in the Dark (DID) method, specifically in order to be able to study self-intoxication
by rodents. The mice we studied here simply did not elect to drink very avidly, and therefore
did not likely reach high BECs. We consider 1.0 mg/ml to be a threshold BEC above which
nearly all mice will show behavioral signs of intoxication (Crabbe et al., 2005). In the
Rhodes et al., (2007) DID study, even when using a DID paradigm, mice with a low
preference for ethanol in the standard 24 hr access test drank very little, reaching an average
BEC = 0.13 mg/ml, a concentration much below the of 1 mg/ml that we consider to be a
pharmacological indicator of intoxication. Nonetheless, in the studies we report here Tg
mice drank significantly less ethanol than WT overall. Although Figure 1A gives the
appearance that their intakes were lower at the highest ethanol concentrations, the statistical
analysis did not support an interaction of genotype and concentration.

Quantitative trait locus (QTL) mapping studies in mice designed to identify genomic regions
where polymorphisms are associated with ethanol preference drinking have employed many
genotypes, most frequently those derived from the C57BL/6J and DBA/2J inbred strains.
These strains are well known to be extreme alcohol-preferers and avoiders, respectively
(Rodgers and McClearn, 1962; Wahlsten et al., 2006). A QTL meta-analysis identified
several QTLs for preference drinking (Belknap and Atkins, 2001) including one centered at
25 centiMorgans (cM) on mouse Chromosome 9. The confidence interval for this QTL
ranges from 10–35 cM (http://www.ohsu.edu/parc/), and the nicotinic receptor cluster maps
to 32 cM, within that confidence interval (http://www.informatics.jax.org/). A recent study
explored the role of nicotinic receptor subunit genes in preference drinking by crossing the
C57BL/6J and DBA/2J strains to create more than 200 reciprocal F2 intercross mice they
then tested for preference for 10% ethanol (Symons et al., 2010). They selected the 86
extreme alcohol preferers and avoiders, who represented the top and bottom 10% of the F2
population, and genotyped them for single nucleotide polymorphisms (SNPs) within each of
six nicotinic subunit genes. Mice with C57BL/6J alleles within the alpha 5, alpha 3 and beta
4 subunit genes drank significantly more than those with DBA/2J alleles. No significant
drinking differences were seen for SNPs within the alpha 4, alpha 7 or beta 2 subunit genes.
The pattern of drinking in four mice showing recombination within the 3-gene cluster
suggested further that the evidence for the alpha 5 subunit was the strongest. To pursue the
possible functionality of the allelic differences, whole brain gene expression of 4 C57BL/6J
and 4 DBA/2J mice was assessed by RT-PCR 6 hr following injection with either saline or 6
g/kg ethanol. Expression of the alpha 3 subunit did not differ significantly. For the alpha 5
subunit gene, the strains did not differ in basal expression levels but ethanol increased
expression in C57BL/6J mice and decreased it in DBA/2J mice. For the beta 4 subunit gene,
there was overall lesser expression in the C57BL/6J mice (Symons et al., 2010). The
expression differences are inconclusive given the small number of samples and the
extremely high dose of ethanol employed. Furthermore, an earlier microarray expression
study of brain tissue from these strains given the same ethanol treatment did not identify
these subunit genes as ethanol-responsive (Treadwell and Singh, 2004). Finally, as the
authors note Symons et al., (2010), evidence of protein level differences would be required
to implicate these genes definitively, because another tightly linked gene on Chromosome 9
might be responsible for these data. Nonetheless, these data overall suggest that the alpha 5
and beta 4 genes may play a role in preference drinking.

We found that several other behavioral responses to acute ethanol injections were unaffected
by over expression of this gene cluster. These included responses to ethanol doses ranging
from low (balance beam) and moderate (hypothermia, rotarod) to anesthetic (loss of righting
reflex and acute withdrawal). Because different behavioral responses to acute ethanol appear
to be influenced largely by discrete genetic factors (Crabbe et al., 2005), our finding an
effect specific to preference drinking is not surprising, and there may be other specific
responses to ethanol on which these genotypes differ.
Two other limitations should be considered. First, we employed a single dose of ethanol for the behavioral assays, a limitation due to the sparse availability of mice. We do not think this should be a major concern, because for most of the effects we studied, the dose-effect curve for mice is rather linear and we were near the expected middle of the DR curves, based on our extensive experience with these tests [for review, see (Crabbe et al., 2005)]. For some tests (LORR, acute withdrawal), a single dose is clearly appropriate because these are both threshold responses. An issue of more plausible concern is the fact that we studied behavioral sensitivity only in mice with a history of prior exposure to alcohol. Thus, the development of tolerance or the influence of some other unspecified carryover influences cannot be discounted. The development of tolerance may be suggested to have played a role in the preference drinking finding, as the genotypes tested in Batch 1, exposed to lower concentrations, appeared to differ more at higher concentrations. However, Batch 2 Tg mice also drank significantly less than WT even when immediately offered 10% ethanol. Separate studies would be needed to assess tolerance in Tg vs. WT mice. We deem tolerance less likely to play a role in the subsequent behavioral tests because of the long intervals separating those tests from either drinking, or each other (at least one week between tests). Although some forms of ethanol tolerance can develop quickly, tolerance generally dissipates with 2–3 days unless ethanol treatment has been severe enough to elicit a state of physical dependence (Kalant, 1998; Kalant et al., 1971). The current studies suggest that Tg and WT mice do not differ in sensitivity to ethanol across a range of tasks and doses, and this in turn predicts that they likely do not differ in tolerance development, but future studies of tolerance will be required to test this hypothesis.

Delivery of the non-specific nAChR antagonist mecamylamine into the ventral tegmental area of rats selectively reduced alcohol preference drinking (Blomqvist et al., 1996) and the associated release of dopamine in the nucleus accumbens (Ericson et al., 1998). Mecamylamine was recently shown to reduce alcohol drinking selectively in mice using the DID binge-drinking paradigm; again, the effects may have been mediated by dopaminergic activity (Hendrickson et al., 2009). In a study with humans, mecamylamine reduced the subjectively pleasurable effects of ethanol (Blomqvist et al., 2002). The same compound also reduced the severity of withdrawal in mice chronically treated with ethanol using a liquid diet (Bhutada et al., 2010). Furthermore, recent pharmacological studies (Chatterjee et al., 2011) have demonstrated that high-affinity partial agonists at α3β4 nAChRs, CP-601932, and PF-4575180, selectively decrease ethanol but not sucrose consumption and operant self-administration following long-term exposure. Interestingly we observed reduced activation of VTA dopaminergic neurons upon nicotine injection in our transgenic mice (Gallego et al., in revision). All these studies suggest a role for alpha 3 and beta 4 receptor subunits in several responses to ethanol.

The fact that we saw parallel results in two transgenic lines gives us some confidence that the results are indeed due to the over expressed human gene cluster. However, we cannot rule out the possible contribution of other genes whose expression or function may have been influenced by the transgene. There is shared genetic risk for dependence on multiple substances, and the nicotinic receptor gene cluster on chromosome 15 harbors multiple polymorphisms associated with this risk. Several association studies with SNPs genotyped across the CHRNA5, CHRNA3, and CHRNβ4 loci on chromosome 15q25.1 of European-American and African-American families have reinforced the importance of variation in the chromosome 15 nicotinic receptor subunit gene clusters for risk of dependence on multiple substances, although the direction of the effects may vary across substances. While an increasing body of evidence suggests that the rs16969968 SNP is associated with multiple substance dependence phenotypes (Erlich et al., 2010; Grucza et al., 2008; Sherva et al., 2010), the mechanism by which minor alleles can increase the risk of alcohol dependence remains to be determined. One potential biological explanation for these effects is based on
reduced receptor function due to the polymorphism differentially impacting the mechanisms through which nicotine and cocaine alter the mesolimbic dopaminergic system (Grucza et al., 2008 and Gallego et al., in revision).

In summary, our study provides preliminary support for a role of the alpha 5, alpha 3, beta 4 nicotinic receptor subunit genes in modulating ethanol intake. Given that the influence on nicotine self-administration was to increase intake over WT controls (Gallego et al., in revision), and the finding that the Tg mice did not show reduced intake of sweet solutions in our studies, we work suggests that their influence is not mediated through rewarding processes generally, but is more selective for ethanol. The reduced VTA dopaminergic activation observed in Tg mice upon nicotine injection (Gallego et al., in revision) may contribute to the observed phenotype. The usual caveats surrounding the use of transgenic models should not be forgotten. Nonetheless, these results add to a growing body of knowledge suggesting the importance of nicotinic receptors in modulating alcohol’s effects, perhaps including alcohol abuse disorders and the co-morbid abuse of alcohol and nicotine.

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Reference List


Overexpression of the CHRNA5/A3/B4 genomic cluster in mice increases the sensitivity to nicotine and modifies its reinforcing effects. AminoAcids. in press.


Figure 1.
EtOH intake and preference drinking. Graphs show the amount of EtOH daily consumption of WT (solid squares) and Tg (open squares) mice (first batch) as g/kg/day (A), the preference as percentage of total fluid intake taken from the ethanol bottle (B), and the total fluid intake (mL/kg/day; WT (open bars) and Tg (solid bars) mice) (C). Saccharin (0.033%) and quinine (0.1 mM) consumption are similarly expressed as mg/kg/day (D) and preference ratio (E). Total intake, saccharin and quinine intakes are averaged across the 4 days test for each alcohol concentration or tastant. Data are presented as mean +/- SEM. See text for statistical analyses. Data shown are from the first of two batches of mice tested.
Figure 2.
Acute sensitivity to EtOH in the balance beam test. The graph shows the number of hind foot slips in WT and Tg mice during transition of the balance beam. Data are presented as mean +/- SEM. See text for statistical analyses.
Figure 3.
Locomotor activity measured in the activity cages. Graphs show horizontal (A) and vertical activity (B) in mice of both lines (L22 and L30). Activity was measured for 10 min before (Baseline) and after EtOH (2.0 g/kg) injection. Data are presented as mean +/- SEM number of beam interruptions. * p = 0.05, *** p < 0.001 versus WT L22 Baseline scores. See text for statistical analyses.
Figure 4.
Performance on the accelerating rotarod. Speed (rpm) of the rotarod at time of fall was measured over 10 consecutive trials in the training session (A) and both before (Baseline) and 30 min after injection of 2.0 g/kg ethanol in the test session (B). Data are presented as mean +/- SEM. See text for statistical analyses.
Figure 5.
Hypothermia induced by ethanol. Body temperature (°C) was measured 30 min prior to EtOH injection (at time 0 min) and 45, 90 and 135 min after EtOH injection (2.0 g/kg). Data are presented as mean +/- SEM. See text for statistical analyses.
Figure 6.
Sensitivity to hypnotic effects of EtOH measured as duration (in min) of loss of the righting reflex (LORR) in WT and Tg mice after EtOH injection (4.0 g/kg). Data are presented as mean +/- SEM. See text for statistical analyses.
Figure 7.
Acute EtOH withdrawal severity measured by handling-induced convulsions (HICs). Graphs show HIC time course in WT and transgenic mice before (Baseline, T0) and after EtOH injection (4.0 g/kg) (A). HICs were scored starting 2 hr after injection and for each hr until hr 12. The area under the curve (AUC) (B) and the peak (C) were derived from the time course data as described in the text. Data are presented as mean +/- SEM. See text for statistical analyses.