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ORIGINAL INVESTIGATION

A role for neuronal nicotinic acetylcholine receptors in ethanol-induced stimulation, but not cocaine- or methamphetamine-induced stimulation

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Abstract
Rationale Cocaine (COC), ethanol (EtOH), and methamphetamine (MA) are widely abused substances and share the ability to induce behavioral stimulation in mice and humans. Understanding the biological basis of behavioral stimulation to COC, EtOH, and MA may provide a greater understanding of drug and alcohol abuse.
Objectives In these studies we set out to determine if neuronal nicotinic acetylcholine receptors were involved in the acute locomotor responses to these drugs, our measure of behavioral stimulation.
Methods A panel of acetylcholine receptor antagonists was used to determine if nicotinic receptors were involved in EtOH- and psychostimulant-induced stimulation. We tested the effect of these drugs in genotypes of mice (FAST and DBA/2J) that are extremely sensitive to this drug effect. To determine which acetylcholine receptor subunits may be involved in this response, relative expression of the α3, α6, β2, and β4 subunit genes was examined in mice selectively bred for high and low response to EtOH.
Results Mecamylamine, but not hexamethonium, attenuated the acute locomotor response to EtOH. The acetylcholine receptor antagonist dihydro-β-erythroidine and methyllycaconitine had no effect on this response. The α6 and β4, but not α3 or β2, subunits of the acetylcholine receptor were differentially expressed between mice bred for extreme differences in EtOH stimulation. Mecamylamine had no effect on psychostimulant-induced locomotor activity.
Conclusions Neuronal nicotinic receptors are involved in EtOH, but not psychostimulant, stimulation. These studies suggest a lack of involvement of some nicotinic receptor subtypes, but more work is needed to determine the specific receptor subtypes involved in this behavior.

Keywords Methamphetamine · Ethanol · Cocaine · Stimulation · Nicotinic acetylcholine receptors · Locomotor activity · Selected lines

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Introduction

Cocaine (COC), ethanol (EtOH), and methamphetamine (MA) are commonly abused substances and share the ability to cause locomotor stimulation in mice (e.g. Crabbe et al. 1983, 1994; Downing et al. 2006; Dudek et al. 1991; Grisel et al. 1997; Marley et al. 1998; Phillips et al. 1998). Sensitivity to drug-induced behavioral stimulation has been suggested to be an important endophenotype for drug abuse (Gabbay 2005). The use of endophenotypes to understand the complex biological basis of behavior has grown in recent years, and the use of these simpler traits has proven more successful than using the clinical diagnosis of alcohol dependence to map genes relevant to alcoholism (Dick et al. 2006).

Genetic correlations have been observed between the acute locomotor response to EtOH and psychostimulants. FAST and SLOW mice were selectively bred for their differential sensitivity to the stimulant effects of EtOH (Crabbe et al. 1987; Phillips et al. 1991, 2002; Shen et al. 1995). FAST mice, which were bred for heightened

sensitivity to the stimulant effects of EtOH, were more sensitive to the stimulant effects of COC and MA compared to SLOW mice (Bergstrom et al. 2003). An independent set of selected lines has been bred for high (HMACT) or low (LMACT) sensitivity to an acute injection of MA. Similar to the FAST and SLOW mouse lines, the HMACT line was more stimulated by EtOH and COC compared to the LMACT line (Kamens et al. 2005, 2006). Furthermore, lines of mice selectively bred for sensitivity to COC's stimulatory effects also differed in response to amphetamine, such that the lines that were more stimulated by COC were more stimulated by amphetamine (Marley et al. 1998). No data exist regarding sensitivity to EtOH in these lines. These data provide support that common genes and neural mechanisms may underlie the acute locomotor responses to these drugs.

The mesolimbic dopamine system has been implicated in drug reward and behavioral activation (Amalric and Koob 1993; Kalivas and Nakamura 1999; Pierce and Kalivas 1997). Elevations in dopamine levels in the nucleus accumbens, one of the key structures in this pathway, have been observed following the administration of EtOH, COC, and MA at doses that stimulate locomotor activity (Carboni et al. 1989; Di Chiara and Imperato 1988; Imperato and Di Chiara 1986; Izawa et al. 2006; Larsson et al. 2002). The mesolimbic dopamine system is a final common pathway through which all three of these drugs produce stimulation; therefore, modulation of this pathway may be important for this behavior. Acetylcholine receptors are expressed in this pathway. One subunit expressed in midbrain neurons is the α3 subunit (Azam et al. 2002; Klink et al. 2001); therefore, α3 subunit-containing receptors are well positioned to modulate dopamine neuron activity and drug-induced stimulation.

Pharmacologic data have implicated α3-containing nicotinic acetylcholine receptors in EtOH-, COC-, and MA-induced behaviors. However, other nicotinic receptor subunit genes are also expressed in dopamine circuits associated with drug-related stimulation. Larsson and colleagues (2002) used a panel of acetylcholine receptor antagonists to determine the role of these receptors in EtOH-induced locomotor stimulation. They showed that mecamylamine, a nonspecific nicotinic acetylcholine receptor antagonist, can attenuate EtOH-induced locomotor stimulation in NMRI outbred mice. In contrast, dihydro-β-erythroidine, an α4β2 receptor antagonist (Khiroug et al. 2004) and methyllycaconitine, an α7 receptor antagonist (Romanelli and Gualtieri 2003), had no effect on EtOH stimulation (Larsson et al. 2002). These data provide evidence against α4β2 and α7 nicotinic receptors in this response. Further, when α-conotoxin MII, an antagonist of α3β2-, β3-, and α6-containing nicotinic receptors was injected into the ventral tegmental area, EtOH-induced stimulation was decreased (Larsson et al. 2004). The involvement of α6-containing nicotinic receptors in this response was ruled out by using α-conotoxin PIA-analog, which specifically blocks α6 receptors; α-conotoxin PIA-analog did not alter the acute response to EtOH (Jerlhag et al. 2006). Together, these data provide support for the involvement of α3β2 nicotinic receptors in EtOH-induced locomotor stimulation, but also leave open the possibility that β3-containing receptors are important.

Further support for the involvement of α3-containing receptors in the behavioral response to EtOH, COC, and MA comes from the use of 18-methoxycoronaridine. 18-Methoxycoronaridine is a potent α3β4 nicotinic antagonist (Glick et al. 2002). 18-Methoxycoronaridine does not appear to alter the acute locomotor response to MA or COC (Szumlinski et al. 2000a, b). However, 18-methoxycoronaridine has been shown to attenuate operant responding for COC and MA (Glick et al. 1996, 2000; Maisonneuve and Glick 2003; Pace et al. 2004) and to decrease EtOH consumption and preference in a two-bottle choice paradigm (Rezvani et al. 1997). To our knowledge, 18-methoxycoronaridine has not been used to study EtOH stimulation. These data provide support for the hypothesis that α3-containing nicotinic acetylcholine receptors are involved in at least some behavioral responses to these drugs.

The goal of these experiments was to evaluate the role of nicotinic acetylcholine receptors in the acute locomotor responses to EtOH, COC, and MA. Furthermore, we were interested specifically in α3-containing acetylcholine receptors. 18-Methoxycoronaridine is not commercially available; therefore, in these studies, we used a panel of nicotinic acetylcholine antagonists: mecamylamine, hexamethonium, dihydro-β-erythroidine, and methyllycaconitine, as well as a molecular approach. While there is evidence for a role of neuronal nicotinic receptors in the acute response to EtOH in outbred mice (Blomqvist et al. 1992; Larsson et al. 2002), we set out to test if these receptors are involved in EtOH-induced stimulation in two genetic models of high EtOH sensitivity, the selectively bred FAST mice and DBA/2J mice, an inbred strain that by chance exhibits robust locomotor stimulation following EtOH (Crabbe et al. 1994; Dudek et al. 1991). We first tested the involvement of neuronal nicotinic receptors in the acute locomotor response to EtOH in FAST and DBA/2J mice. We hypothesized that mecamylamine would attenuate EtOH-induced stimulation, but that hexamethonium, a nonspecific nicotinic acetylcholine receptor antagonist that does not cross the blood brain barrier, would not.

A more detailed investigation into which type of nicotinic receptors may be involved in the acute locomotor response to EtOH was performed in FAST mice because they were selectively bred for this trait. We hypothesized that dihydro-β-erythroidine and methyllycaconitine would

have no effect on EtOH stimulation. Because our hypothesis was that α3-containing nicotinic receptors are involved in EtOH stimulation, we also utilized quantitative real-time PCR (qRT-PCR) to examine potential differences between the FAST and SLOW lines in α3 gene expression, as well as in the expression of the α6, β2, and β4 subunit genes (subunits that form functional receptors with α3). A difference in expression would suggest that selective breeding for differential EtOH sensitivity simultaneously altered factors involved in the expression of this (these) gene(s).

Finally, we wanted to examine if the effects of nicotinic receptors on EtOH-induced stimulation generalize to psychostimulants. DBA/2J mice are extremely sensitive to COC- and MA-induced locomotor stimulation (Grisel et al. 1997; Phillips et al. 1998). To test the role of nicotinic receptors in the acute locomotor response to these drugs, we used the nonspecific antagonist mecamylamine. We hypothesized that mecamylamine would attenuate COC- and MA-induced stimulation.

Methods

Mice were maintained in the Portland Veterans Affairs Veterinary Medical Unit. Mice were housed 2–5 per cage in standard plastic cages with Bed-o cobs' (The Andersons, Maumee, OH, USA) lining. Animals had food (Purina Laboratory Rodent Chow #5001; Purina Mills, St. Louis, MO, USA) and water available ad libitum. Mice were kept in colony rooms on a 12-h light–dark cycle (lights on at 0600 hours) with the temperature maintained at 21±2°C. All procedures were approved by the Portland Veterans Affairs Medical Center Institutional Animal Care and Use Committee and were consistent with the *Guide for the Care and Use of Laboratory Animals* (National Research Council 1996).

All mice were 50–101 days old and experimentally naïve at the start of testing. Mice from a single cage were distributed across groups to avoid all mice from a single family/cage from being assigned to a single drug group.

Subjects

FAST mice Male and female FAST-1, SLOW-1, FAST-2, and SLOW-2 mice were used for these experiments. The selection of the FAST and SLOW lines has been described in detail elsewhere (Crabbe et al. 1987; Phillips et al. 1991, 2002; Shen et al. 1995). Briefly, the FAST and SLOW mice were created through a selective breeding process starting from the heterogeneous HS/Ibg stock (McClearn et al. 1970). Mice were bred for 37 generations for their high (FAST) or low (SLOW) sensitivity to the acute locomotor stimulant effects of EtOH. First litter offspring of each generation were tested for their response to EtOH (1.5–2 g/kg) for 4 min beginning 2 min after injection in circular open fields (LVE model PAC-001; Lehigh Valley, PA, USA) on day 1. Testing was repeated after an injection of saline on day 2. The difference in locomotor activity was used as the selection criterion (day 1 EtOH response–day 2 saline response). The breeders of the FAST lines were chosen for their extremely high activity scores, while the breeders of the SLOW lines were chosen for their low scores. After 37 generations, selection was relaxed and breeders were arbitrarily chosen within each line. The lines continued to remain divergent in their response to EtOH after selection pressure had been relaxed (Phillips et al. 2002). Two replicated (1 and 2) lines in each direction were concurrently bred and maintained as independent breeding populations. Mice used in the behavioral studies came from $S_{37}G_{75}$–G_{81}, while mice for the qRT-PCR experiment came from $S_{37}G_{84}$–G_{85} (where S_{xx} refers to the number of selected generations and G_{yy} refers to total number of generations that have elapsed since selection began). For pharmacology experiments, only FAST mice were used because we were interested in testing the effect of nicotinic receptor antagonist on EtOH's stimulant response and SLOW mice show no locomotor activation or show locomotor depression following EtOH (Palmer et al. 2002; Phillips et al. 2002; Shen et al. 1995). Only females were used for the hexamethonium experiment because they were more available at the time of testing and because sex was not found to interact with other independent variables in the other experiments.

DBA/2J mice Male DBA/2J mice were either bred at the Portland Veterans Affairs Medical Center or obtained from The Jackson Laboratory (Bar Harbor, ME, USA). When mice were obtained from The Jackson Laboratory, they acclimated to our animal facility for at least 2 weeks prior to being tested. Mice bred in our facility were derived from DBA/2J mice obtained from The Jackson Laboratory; new breeder pairs are obtained each year to avoid spontaneous mutations arising in our colony.

Drugs

Cocaine HCl, dihydro-β-erythroidine HBr, hexamethonium Br, mecamylamine HCl, MA HCl, and methyllycaconitine citrate were purchased from Sigma (St. Louis, MO, USA). Two hundred proof ethyl alcohol was obtained from Pharmco (Brookfield, CT, USA). All nicotinic antagonists were dissolved in physiological saline (0.9% NaCl; Baxter Healthcare, Deerfield, IL, USA) to appropriate concentrations, while EtOH was diluted in saline to 20% v/v. The injection volume of all nicotinic antagonists was 10 ml/kg body weight.

Apparatus

Eight AccuScan automated activity monitors (Columbus, OH, USA) were used to measure locomotor activity. The monitors have eight photocell beams along two sides and detectors on the opposite sides. The activity monitors record the number of photocell breaks, which is translated into distance traveled (in cm). Inside each monitor sat a 40×40×30-cm (l × w × h) clear acrylic test chamber. The chamber and monitor were encased by a black insulated acrylic chamber to separate the testing environment from the external environment. Inside the chamber was a fan to provide background noise and an 8-W fluorescent light for illumination. Because mice were tested between 0800 and 1600 hours, the light was on for consistency with their normal light/dark cycle.

Procedure

Mice were moved to the testing room 45–60 min prior to the start of the experiment to allow time to acclimate. Animals were weighed and placed into individual holding cages while syringes were prepared (for up to 10 min prior to testing). FAST mice were first injected with one dose of one of four pretreatment drugs, dihydro-β-erythroidine (0, 0.5, 1, 1.5, or 2 mg/kg), hexamethonium (0, 2, 4, 6, or 8 mg/kg), mecamylamine (0, 1, 2, 3, 4, or 6 mg/kg), or methyllycaconitine (0, 1, 2, 3, or 4 mg/kg). Ten minutes later they received a second injection of either EtOH (2 g/kg) or saline. The 2-g/kg dose of EtOH was chosen because FAST-1 and FAST-2 mice show their greatest stimulant response to this EtOH dose (Palmer et al. 2002) and it was the dose used for most selection generations. DBA/2J mice were treated with mecamylamine (0, 1, 2, 3, or 4 mg/kg) or hexamethonium (0, 2, 4, 6, or 8 mg/kg) immediately prior to an injection of EtOH (1.5 g/kg), COC (10 mg/kg), or MA (2 mg/kg). These doses were chosen because they were known to produce robust behavioral activation in this strain (Dudek et al. 1991; Phillips et al. 1998). The pretreatment times and doses of nicotinic antagonist drugs were based on prior work examining the effects of these drugs on behaviors (Blomqvist et al. 1992; Damaj et al. 2003; Gommans et al. 2000; Larsson et al. 2002), as well as pilot testing in our laboratory. Immediately following the second injection, animals were placed into the center of the activity monitor where locomotor activity was measured for 15 (EtOH) or 30 min (COC and MA) in 5-min epochs. At the end of the test session, animals that received EtOH had a 20-μl blood sample taken from the retro-orbital sinus to determine blood EtOH concentrations (BEC). The blood was put into tubes containing 50 μl $ZnSO_4$ (5%) and placed on ice until processing, at which time 50 μl Ba $(OH)_2$ (0.3 N) and 300 μl dH_2OH were added to the tube. Following centrifugation, supernatant was removed and BECs were determined by gas chromatography (Agilent 6890N) following procedures standard in our laboratory (Boehm et al. 2000).

RNA isolation and qRT-PCR

Tissue and RNA preparation Experimentally naïve male and female FAST and SLOW mice were cervically dislocated before being decapitated; brains were removed and immediately frozen in isopentane. Brains were stored frozen at −80° C until they could be processed. RNA was extracted using a guanidinium isothiocyanate–phenol–chloroform extraction method (Chomczynski and Sacchi 1987, 2006). Briefly, brains were homogenized in RNA Stat-60 (Tel-Test, Friendswood, TX, USA) using a polytron tissue homogenizer (Brinkmann, Westbury, NY, USA). Chloroform (0.2 ml) was mixed with 1 ml of brain homogenate and was incubated at room temperature. The homogenate was centrifuged and RNA from the aqueous phase was removed and put into a new tube. RNA was precipitated out with the addition of isopropanol (0.5 ml) and allowed to sit at room temperature for 10 min before centrifugation. The RNA pellet was then washed with 0.5 ml 75% EtOH and centrifuged. The RNA was allowed to air dry before being resuspended in 50 μl diethylpyrocarbonate (DEPC)-treated water. The sample was cleaned of DNA contamination using the DNA-Free RNA Kit (Zymo Research, Orange, CA, USA), using the manufacturer's specifications. Briefly, the RNA was incubated for 15 min at 37°C with RNase-free DNase I and 10× DNase I buffer to degrade DNA from the sample. The RNA was mixed with a RNA binding buffer before being put onto a Zymo-Spin IC column. The column was washed twice and RNA was eluted with the addition of DEPC water directly onto the column. RNA quality was assessed with a spectrophotometer (Eppendorf, Hamburg, Germany; ratio 260/280 nm=1.8–2; Chomczynski and Sacchi 2006) and confirmed by gel electrophoresis.

Reverse transcription and qRT-PCR One microgram of total RNA was reverse transcribed using a high-capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). To each RNA sample, 10 μl 10× RT buffer, 4 μl 25× deoxyribonucleotide triphosphates, 10 μl 10× Random Primers, and 5 μl MultiScribe RT was added. The mixture was incubated at 25°C for 10 min followed by 37°C for 2 h in an iCycler (Bio-Rad, Hercules, CA, USA).

Predesigned TaqMan gene expression assays (Applied Biosystems) were obtained for the *Chrna3*, *Chrna6*, *Chrnb2*, *Chrnb4* (the α3, α6, β2, and β4 subunits of the acetylcholine receptor, respectively) genes and the control *Hprt1* (hypoxanthine guanine phosphoribosyl transferase 1) gene. *Hprt1* was used as the control gene because it has been validated as a reference gene in studies using mouse

models of neurological diseases (e.g., Meldgaard et al. 2006). qRT-PCR reactions were run using an iCycler (Bio-Rad). Five microliters of cDNA was added to 10 μl TaqMan Universal Master Mix, 1 μl of the gene specific primers, 0.4 μl fluorescein, and 3.6 μl DEPC water. The reaction began at 50°C for 2 min followed by 95°C for 10 min; the sample then went through 40 cycles of denaturing at 95°C for 15 s followed by 1 min at 60°C that allowed for the primers to anneal and for amplification. All samples were run in triplicate and the average crossing threshold (C_t) was used as a measure of relative quantification for the *Chrna3*, *Chrna6*, *Chrnb2*, *Chrnb4*, and *Hprt1* genes. For analysis, the average C_t of the control *Hprt1* gene was subtracted from the C_t of the nicotinic acetylcholine subunit gene. Relative expression based on the $\Delta\Delta$ C_t method was defined as 2 to the power of the negative average expression of the FAST mice minus each individual value (this was done independently for each replicate; Livak and Schmittgen 2001).

Analyses

Statistica (StatSoft, Tulsa, OK, USA) was used for all statistical analyses. Data were analyzed by using factorial analysis of variance (ANOVA) with the alpha level set at 0.05. Depending on the study, a number of independent variables were used, including replicate, line, sex, pretreatment drug dose, and challenge drug dose. Interactions involving three or more factors were broken down using ANOVAs with fewer factors; two-way interactions were analyzed using simple effects and Newman–Keuls test for post hoc comparisons.

Results

FAST mice

Mecamylamine attenuated EtOH stimulation in both the FAST-1 and FAST-2 lines of mice (Fig. 1). For all EtOH experiments, data from the last 10 min of the 15-min test are presented because the antagonist effects were strongest during this time; however, similar results were obtained for all time periods examined. Data were analyzed using a four-way ANOVA with replicate, sex, mecamylamine dose, and EtOH dose as factors. There was a main effect of replicate ($F_{1,\ 498}$=11.2, p<0.001) and a significant replicate x EtOH dose interaction ($F_{1,\ 498}$=85.8, p<0.001); therefore, all further analyses were performed separately on the data from the FAST-1 and FAST-2 lines. Because there were no main effects or interactions with sex, male and female data were combined.

For FAST-1 mice, there was a significant pretreatment × EtOH dose interaction ($F_{5,\ 272}$=3.7, p<0.01); pretreatment with mecamylamine decreased EtOH-induced stimulation but did not affect saline activity. There was a significant simple main effect when data from groups that received an EtOH injection were included in the analysis; there was less stimulation in the groups that received 3, 4, or 6 mg/kg mecamylamine compared to the saline-pretreated animals or animals that received a pretreatment of 1 mg/kg mecamylamine. Additionally, mice that were pretreated with 6 mg/kg mecamylamine prior to an EtOH injection showed reduced stimulation compared to animals that received a 2-mg/kg mecamylamine pretreatment (p values<0.01). Mecamylamine did not significantly decrease activity following saline, as indicated by a nonsignificant simple main effect for the saline-treated groups. BECs were missing for 17 animals due to blood processing errors. This resulted in data missing from three mice from each of the saline and 1-, 2-, and 6-mg/kg mecamylamine pretreatment groups and four data points missing from the 3- and 4-mg/kg mecamylamine pretreatment groups. Even with this data loss, the smallest number of blood sample data points in a single group was 21. There was no effect of pretreatment with mecamylamine on BECs 15 min post-EtOH injection in FAST-1 mice (mean ± SEM: 2.26±0.07, 2.39±0.08, 2.16±0.08, 2.36±0.06, 2.19±0.09, and 2.21±0.07 for saline, 1, 2, 3, 4, and 6 mg/kg mecamylamine, respectively).

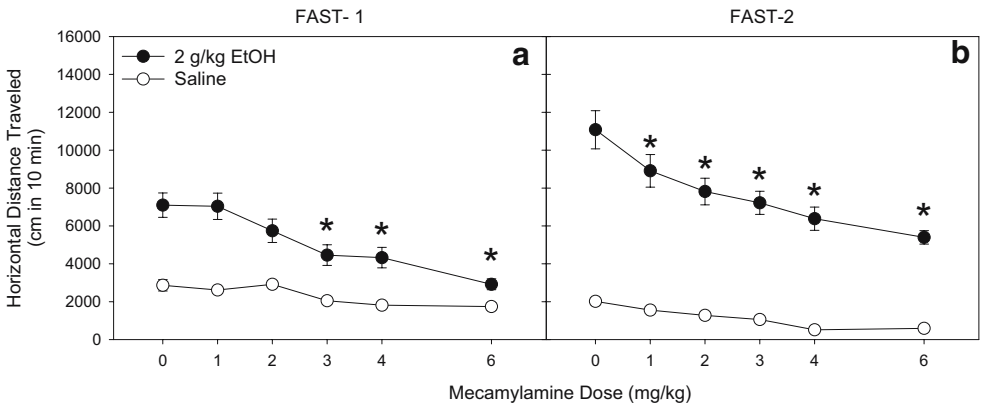

Fig. 1 Mecamylamine attenuated EtOH-induced stimulation in **a** FAST-1 and **b** FAST-2 mice. Data (mean ± SEM; some *error bars* may be hidden by the *symbols*) are from the last 10 min of a 15-min test. N=20–26 per group. *Asterisks*, significantly different from the EtOH control group

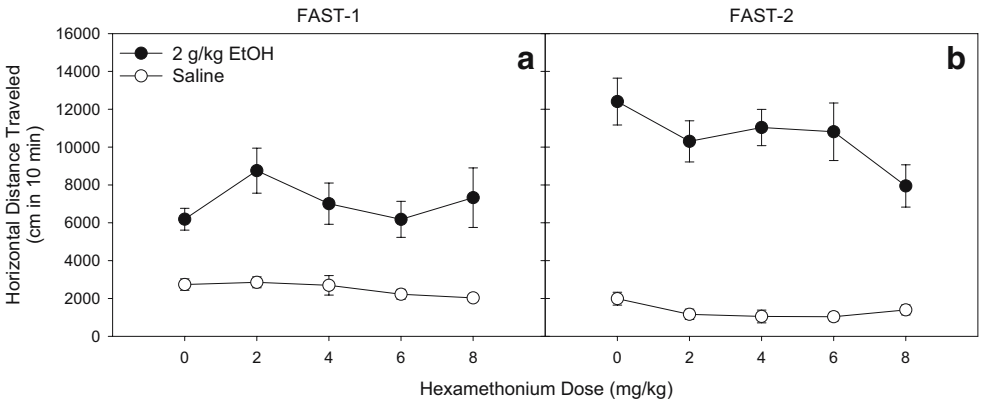

Fig. 2 Hexamethonium did not affect EtOH (2 g/kg) stimulated activity in **a** FAST-1 or **b** FAST-2 mice. Data (mean ± SEM; some *error bars* may be hidden by the *symbols*) are from the last 10 min of a 15-min test. FAST-1 and FAST-2 mice were stimulated by EtOH ($F_{1,\ 62}$=89.3, p< 0.001; $F_{1,\ 60}$=250.1, p<0.001, respectively), but EtOH dose did not interact with hexamethonium dose. N=5–14 per group

A significant pretreatment × EtOH dose interaction was also observed in FAST-2 mice ($F_{5,\ 250}$=4.0, p<0.01). EtOH-induced stimulation was reduced in all groups of animals receiving pretreatment with mecamylamine compared to groups that received a pretreatment with saline. Pretreatment with 6 mg/kg mecamylamine reduced EtOH stimulation to a greater extent than pretreatment with 2 mg/kg mecamylamine (p values<0.05). Pretreatment with mecamylamine did not significantly alter saline activity; the simple main effect for the saline groups was not significant. Pretreatment with mecamylamine did not alter BECs in FAST-2 mice (mean ± SEM: 2.22±0.09, 2.15±0.09, 2.31±0.06, 2.22±0.08, 2.18±0.08, and 2.34± 0.06 for saline, 1, 2, 3, 4, and 6 mg/kg mecamylamine, respectively).

None of the other nicotinic receptor antagonists tested significantly reduced EtOH-induced stimulation in FAST mice (Figs. 2, 3, and 4; see figure legends for additional statistics). In all studies, FAST mice were significantly more stimulated when given EtOH as compared to saline, as indicated by a main effect of EtOH dose. Pretreatment with hexamethonium, methyllycaconitine, or dihydro-β-erythroidine did not significantly alter the acute locomotor response to EtOH or saline. None of these nicotinic antagonists altered EtOH levels observed 15 min post-EtOH injection in either the FAST-1 or FAST-2 lines of mice (data not shown).

Chrna6 and *Chrnb4* were differentially expressed between the FAST and SLOW mice, but *Chrna3* and *Chrnb2* were not (Table 1). There was significantly more *Chrna6* expression in SLOW mice compared to FAST mice, as indicated by a main effect of line ($F_{1,\ 40}$=4.2, p<0.05), but no other significant main effects or interactions with replicate or sex were detected. Statistical analysis of *Chrnb4* expression data also showed greater expression in SLOW mice compared to FAST mice, as indicated by a significant main effect of line ($F_{1,\ 40}$=11.4, p<0.05). Further, male mice were found to have significantly more *Chrnb4* expression than female mice ($F_{1,\ 40}$=4.7, p<0.05; 1.20±0.06 and 1.06±0.04, respectively), but sex did not interact with any other factor, and there were no significant effects associated with replicate. For *Chrnb2* expression, the lines did not differ, but there were significant main effects of replicate and sex [replicate 1 (1.10±0.05) > replicate 2 (0.98±0.03), $F_{1,\ 40}$=5.2, p<0.05; male (1.11± 0.04) > female (0.96±0.04), $F_{1,\ 40}$=2.3, p<0.01]. There were no significant main effects or interactions when *Chrna3* expression data were analyzed.

DBA/2J Mice

Ethanol Similar to the results for FAST mice, mecamylamine completely abolished EtOH (1.5 g/kg) stimulation at the 3- and 4-mg/kg doses in DBA/2J mice (Fig. 5). Because

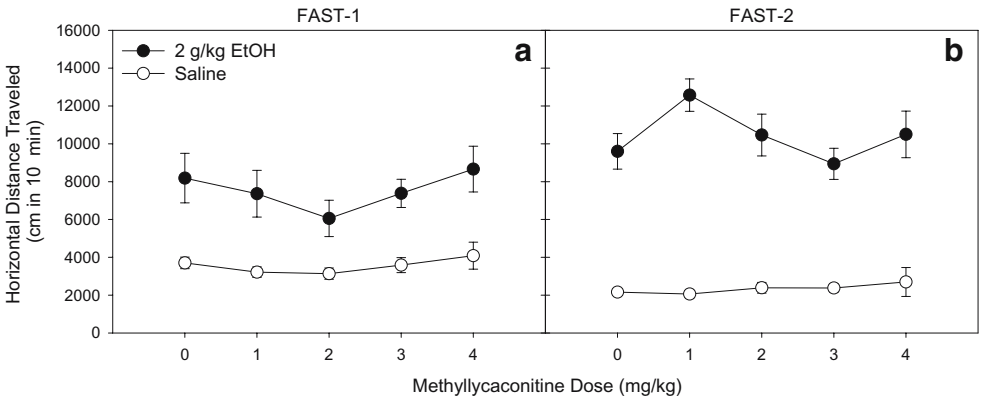

Fig. 3 Methyllycaconitine did not affect EtOH (2 g/kg) stimulated activity in **a** FAST-1 or **b** FAST-2 lines. Shown is the mean (±SEM; some *error bars* may be hidden by the *symbols*) of the last 10 min of a 15-min test. FAST-1 and FAST-2 mice were stimulated by EtOH ($F_{1,\ 101}$=54.7, p<0.001; $F_{1,\ 112}$= 270.5, p<0.001, respectively), but EtOH dose did not interact with methyllycaconitine dose. N=10–13 per group

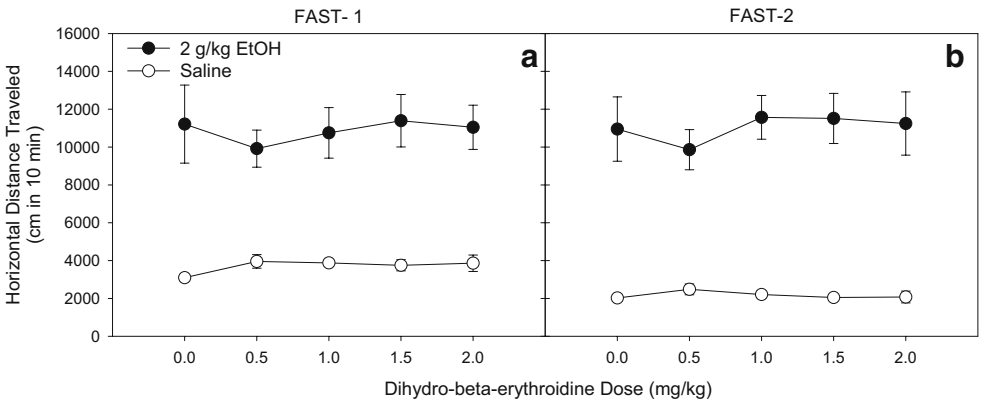

Fig. 4 Dihydro-β-erythroidine did not affect EtOH (2 g/kg) stimulated activity in **a** FAST-1 or **b** FAST-2 lines. Shown is the mean (±SEM; some *error bars* may be hidden by the *symbols*) of the last 10 min of a 15-min test. FAST-1 and FAST-2 mice were stimulated by EtOH ($F_{1,\ 90}$ =123.8, $p<0.001$; $F_{1,\ 90}$=245.8, $p<0.001$, respectively), but this did not interact with dihydro-β-erythroidine dose. N=9–12 per group

there were no significant effects of sex in FAST mice, only male DBA/2J mice were tested. A two-way ANOVA provided evidence of a significant pretreatment × EtOH dose interaction ($F_{4,\ 79}$=3.6, $p<0.05$). There was a significant simple main effect when data from the groups of mice that were injected with EtOH were included in the analysis; EtOH-induced stimulation was decreased in mice that received all doses of mecamylamine compared to the saline-pretreated animals. The simple main effect for the groups of mice receiving saline was not significant, indicating that mecamylamine did not significantly decrease basal locomotor activity. The saline and EtOH treatment groups differed when given saline pretreatment or pretreatment with 1 or 2 mg/kg mecamylamine, but not 3 or 4 mg/kg mecamylamine, providing evidence that these high doses of mecamylamine completely abolished EtOH-induced stimulation in DBA/2J mice. Pretreatment with mecamylamine did not change BECs 15 min post-EtOH injection (mean ± SEM: 1.79±0.06, 1.67±0.12, 1.61±0.09, 1.77±0.05, and 1.60±0.14 for saline, 1, 2, 3, and 4 mg/kg mecamylamine, respectively).

Hexamethonium did not attenuate EtOH-induced stimulation in DBA/2J mice (Fig. 6). There was a main effect of treatment; mice receiving EtOH were more stimulated than mice that received saline ($F_{1,\ 109}$=42.9, $p<0.001$), but there were no main effects or interactions with hexamethonium pretreatment. Likewise, pretreatment with hexamethonium did not alter BEC levels 15 min post-EtOH injection (data not shown).

Cocaine Mecamylamine did not significantly attenuate COC-induced locomotor stimulation (Fig. 7). To be consistent with the EtOH results, we present data from minutes 5–15 of the 30-min test. The results did not change when other time points were examined. There was a main effect of pretreatment ($F_{4,\ 126}$=7.1, $p<0.001$) and a main effect of COC dose ($F_{1,\ 126}$=300.3, $p<0.001$), but the pretreatment × COC dose interaction was not significant. The 2-, 3-, and 4-mg/kg doses of mecamylamine produced a nonspecific decrease in locomotor activity compared to the saline and 1-mg/kg dose groups when data were collapsed on the COC dose factor. COC enhanced locomotor activity compared to mice that received a saline injection.

Methamphetamine Pretreatment with mecamylamine did not significantly attenuate MA-induced stimulation (Fig. 8) during minutes 5–15 of the 30-min test. Similar results were observed at all other time points. MA (2 mg/kg) produced robust stimulation in DBA/2J mice ($F_{1,\ 124}$=73.6, $p<0.001$), but there was no main effect of the pretreatment or a pretreatment × MA dose interaction.

Discussion

In the present studies, we set out to determine if neuronal nicotinic acetylcholine receptors were involved in the acute

Table 1 Expression of the *Chrna3*, *Chrna6*, *Chrnb2*, and *Chrnb4* genes in FAST-1, SLOW-1, FAST-2, SLOW-2, FAST, and SLOW mice

	FAST-1	SLOW-1	FAST-2	SLOW-2	FAST	SLOW
Chrna3	1.02±0.06	1.23±0.11	1.10±0.14	0.97±0.07	1.06±0.07	1.10±0.07
Chrna6	1.02±0.05	1.23±0.10	1.03±0.08	1.21±0.13	1.02±0.04	1.22±0.08[a]
Chrnb2	1.03±0.07	1.17±0.06	1.00±0.03	0.95±0.05	1.02±0.04	1.06±0.04
Chrnb4	1.02±0.06	1.37±0.08	1.01±0.04	1.12±0.08	1.02±0.04	1.24±0.06[a]

Mean (±SEM) relative *Chrna3*, *Chrna6*, *Chrnb2*, and *Chrnb4* gene expression in the FAST and SLOW mice. N=12 per line and replicate
[a] Significantly greater expression compared to FAST mice

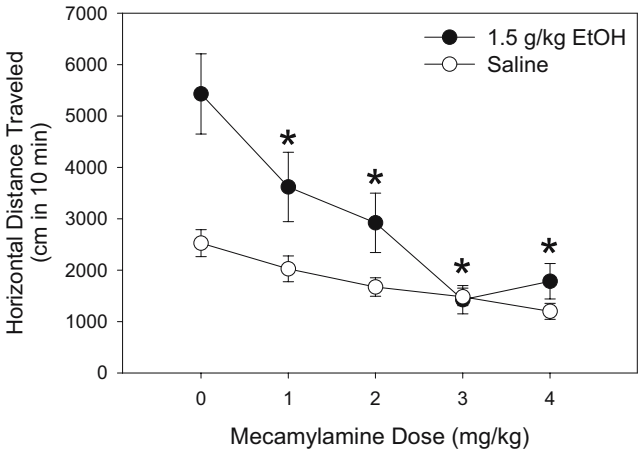

Fig. 5 Mecamylamine attenuated EtOH-induced stimulation in DBA/2J mice. Data (mean ± SEM; some *error bars* may be hidden by the *symbols*) are from the last 10 min of a 15-min test. *N*=8–9 per group. *Asterisks*, significantly different from the EtOH control group

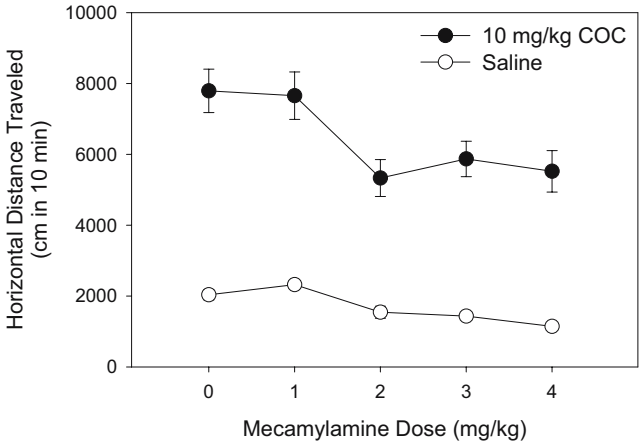

Fig. 7 Mecamylamine did not affect COC (10 mg/kg) stimulated activity in DBA/2J mice. Shown is the mean (±SEM; some *error bars* may be hidden by the *symbols*) of min 5–15 of a 30-min test. *N*=13–14 per group

locomotor responses to EtOH, COC, and MA in mice. The nonspecific nicotinic receptor antagonist mecamylamine had no effect on COC- or MA-induced locomotor activity. However, mecamylamine was able to partially attenuate the acute locomotor response to EtOH in FAST mice and completely abolish this response in DBA/2J mice. Additional work was done in FAST mice using a panel of acetylcholine receptor antagonists to examine which receptors were involved in this effect of mecamylamine. While our data appear to rule out the involvement of some nicotinic receptors, more work is needed to determine which acetylcholine receptors are involved in this response. Gene expression analyses provide hypotheses on which subunits may be involved in this response, but further work is needed to address these hypotheses.

Our inability to detect a significant effect of mecamylamine on MA-induced activity in DBA/2J mice is consistent with prior literature. In rats, the acetylcholine receptor antagonists mecamylamine and dihydro-β-erythroidine had no effect on the acute locomotor response to amphetamine (Schoffelmeer et al. 2002). Similarly, 18-methoxycoronaridine and methyllycaconitine did not alter the acute locomotor response to MA (Escubedo et al. 2005; Szumlinski et al. 2000a). These data support the idea that nicotinic receptors are not involved in the acute locomotor response to amphetamines.

The results involving nicotinic acetylcholine receptors in COC stimulation are less consistent. Although we found no effect of mecamylamine on COC-induced locomotor activity, other studies have implicated nicotinic receptors in this response. Mice lacking the α4 subunit showed a heightened locomotor response to an acute injection of COC (Marubio et al. 2003). Consistent with our results, the acute locomotor response to COC in mice lacking the β2

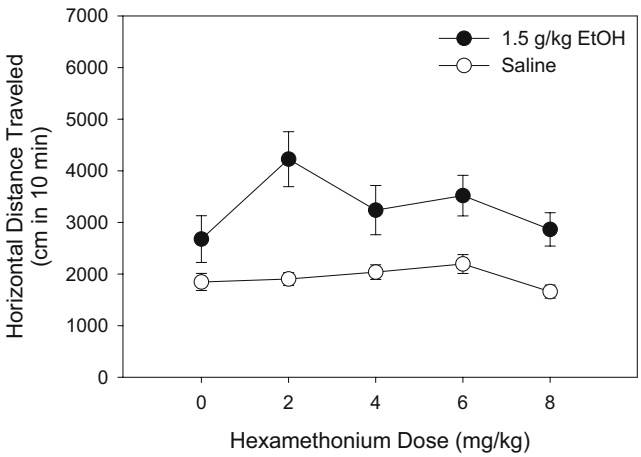

Fig. 6 Hexamethonium did not affect EtOH (1.5 g/kg) stimulated activity in DBA/2J mice. Shown is the mean (±SEM; some *error bars* may be hidden by the *symbols*) of the last 10 min of a 15-min test. *N*=11–12 per group

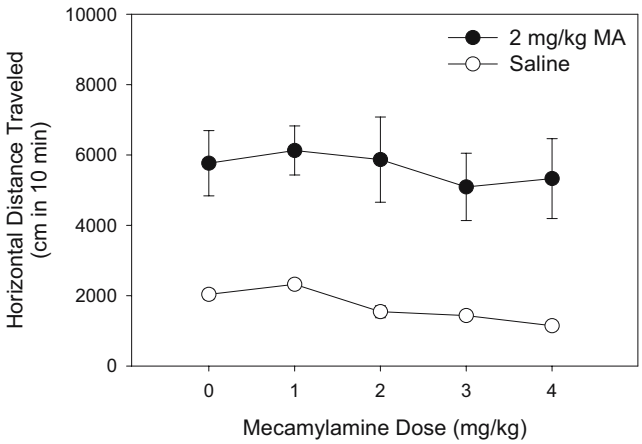

Fig. 8 Mecamylamine did not affect MA (2 mg/kg) stimulated activity in DBA/2J mice. Shown is the mean (±SEM; some *error bars* may be hidden by the *symbols*) of minutes 5–15 of a 30-min test. *N*=13–14 per group

subunit was indistinguishable from that of wild-type mice (Zachariou et al. 2001). Additionally, 18-methoxycoronaridine had no effect on COC stimulation (Szumlinski et al. 2000b).

In contrast to COC, the results obtained in the FAST and DBA/2J mice regarding the involvement of neuronal nicotinic receptors in EtOH-induced stimulation are consistent with findings from NMRI outbred mice. That central nicotinic receptors have been implicated in this response in outbred, inbred, and selectively bred mice provides strong support for the involvement of these receptors in EtOH stimulation. This is supported by no effect of pretreatment with hexamethonium but a reduction in EtOH-induced stimulation following an injection of mecamylamine (Blomqvist et al. 1992). Mecamylamine appeared to decrease baseline locomotor activity, although in no case was this effect statistically significant. In contrast, the significant effects in EtOH-treated mice provide evidence of the involvement of these receptors in EtOH-stimulated activity. However, the possibility of a floor effect in the saline-treated groups must be entertained. Some concerns are assuaged by examination of the figures in which it can be seen that there are doses of mecamylamine that significantly attenuate response to EtOH without having an apparent effect on the activity of saline-treated mice.

Data in NMRI and FAST mice begin to address which nicotinic acetylcholine receptors may be involved in this behavior. The acute locomotor response to EtOH in NMRI mice has been shown to be attenuated by mecamylamine, but not methyllycaconitine or dihydro-β-erythroidine (Larsson et al. 2002). In this paper, we report similar findings in FAST mice that were selectively bred for extreme EtOH-induced stimulation. Because dihydro-β-erythroidine and methyllycaconitine do not influence this behavior, this provides evidence against the involvement of α4β2 and α7 acetylcholine receptors, respectively. In NMRI outbred mice, EtOH-induced stimulation has been shown to be attenuated by the α-conotoxin MII, providing evidence that α3β2-, β3-, or α6-containing nicotinic receptors may be involved in this response (Larsson et al. 2004).

Nicotinic acetylcholine receptors containing an α6 subunit are located in the mesolimibic dopamine pathway and have recently been found to modulate dopamine release (Salminen et al. 2004). The localization and function of these receptors make α6 an interesting candidate as a modulator of this response, but there is pharmacological evidence against the involvement of α6-containing receptors. In NMRI outbred mice, the α6 specific conotoxin, α-conotoxin PIA-analog, did not significantly alter the acute locomotor response to EtOH (Jerlhag et al. 2006). In contrast, there was greater α6 expression in SLOW mice compared to FAST mice, providing at least some evidence that these receptors may be involved in EtOH-stimulation in the FAST and SLOW mice. Furthermore, in our data we also found increased β4 gene expression in the SLOW mice compared to FAST mice. Future work should examine the role of acetylcholine receptors containing these subunits in the FAST and SLOW mice in specific brain regions.

The β3 subunit of the nicotinic acetylcholine receptor forms functional receptors when combined with other α/β pairs such as α3β4, α6β2, and α4β2 (Broadbent et al. 2006; Groot-Kormelink et al. 1998). This subunit has been shown to modulate nicotinic receptor kinetics but does not appear to change agonist binding (Boorman et al. 2003). Mice carrying a null mutation of the β3 subunit have enhanced baseline locomotor activity (Cui et al. 2003), but to our knowledge these mice have not been tested for drug-induced activity.

The role of α3-containing acetylcholine receptors in EtOH-induced stimulation remains to be determined. To our knowledge, 18-methoxycoronaridine has not been tested for EtOH stimulation but does decrease EtOH drinking (Rezvani et al. 1997). In the FAST and SLOW mice, we were not able to detect expression differences in *Chrna3*, but this does not rule out the involvement of receptors containing this subunit. One possible reason why we may not have been able to detect a gene expression difference is that we used a whole brain preparation in this initial investigation. Gene expression assays using brain regions known to contain this subunit may provide different results.

Not only have neuronal nicotinic receptors been implicated in the acute locomotor response to EtOH in mice, but in humans, mecamylamine decreased reported ratings of EtOH-induced stimulation (Young et al. 2005) and euphoria (Chi and de Wit 2003). Together, these data provide support for the idea that nicotinic receptors may be involved in the acute response to EtOH in both humans and animal models and may provide important information about the neural mechanisms involved in alcohol's effects.

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