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Nicotine enhances the hypnotic and hypothermic effects of alcohol in the mouse

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Abstract

Background—Ethanol and nicotine abuse are two leading causes of preventable mortality in the world, but little is known about the pharmacological mechanisms mediating co-abuse. Few studies have examined the interaction of the acute effects of ethanol and nicotine. Here, we examine the effects of nicotine administration on the duration of ethanol-induced loss of righting reflex (LORR) and characterize the nature of their pharmacological interactions in C57BL/6J mice.

Methods—We assessed the effects of ethanol and nicotine and the nature of their interaction in the LORR test using isobolographic analysis after acute injection in C57BL/6J male mice. Next, we examined the importance of receptor efficacy using nicotinic partial agonists varenicline and sazetidine. We evaluated the involvement of major nAChR subtypes using nicotinic antagonist mecamylamine and nicotinic $\alpha 4$ and $\alpha 7$ knockout mice. The selectivity of nicotine's actions on ethanol-induced LORR was examined by testing nicotine's effects on the hypnotic properties of ketamine and pentobarbital. We also assessed the development of tolerance after repeated nicotine exposure. Lastly, we assessed if the effects of nicotine on ethanol-induced LORR extends to hypothermia and ethanol intake in the Drinking in the Dark (DID) paradigm.

Results—We found that acute nicotine injection enhances ethanol's hypnotic effects in a synergistic manner and that receptor efficacy plays an important role in this interaction. Furthermore, tolerance developed to the enhancement of ethanol's hypnotic effects by nicotine after repeated exposure of the drug. $\alpha 4^*$ and $\alpha 7$ nAChRs seem to play an important role in nicotine-ethanol interaction in the LORR test. In addition, the magnitude of ethanol-induced LORR enhancement by nicotine was more pronounced in C57BL/6J than DBA/2J mice. Furthermore, acute nicotine enhanced ketamine and pentobarbital hypnotic effects in the mouse.

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Finally, nicotine enhanced ethanol-induced hypothermia but decreased ethanol intake in the DID test.

Conclusion—Our results demonstrate that nicotine synergistically enhances ethanol-induced LORR in the mouse.

Keywords

ethanol; nicotine; mice; LORR

Introduction

Ethanol abuse and nicotine use are two leading causes of preventable mortality in the world. Ethanol abuse is responsible for nearly 80,000 deaths each year in the United States while tobacco products are responsible for nearly 440,000 deaths each year in the United States (Centers for Disease Control 2005). There is a high rate of co-occurrence between smoking and ethanol with 70–90% of alcoholic subjects also smokers (Sher et al., 1996). This high co-morbidity of use increases the difficulty of achieving long-term abstinence with either drug (Larsson et al. 2004). Current evidence strongly points to the existence of complex biological interactions between the drugs, which suggests that there may be common biological mechanisms mediating co-abuse (True et al. 1999; Davis and de Fiebre 2006; Schlaepfer et al. 2008). Additionally, there is evidence of both synergistic and antagonistic interactions between the two drugs. For example, exposure to ethanol and nicotine together results in additive dopamine release in the nucleus accumbens of rats (Schlaepfer et al. 2008). In contrast, nicotine attenuates ethanol-induced ataxia (Taslim et al. 2008, 2011). Mecamylamine, a nonselective antagonist of nicotinic acetylcholine receptors (nAChRs), has been shown to decrease ethanol-induced dopaminergic neuron activation and ethanol intake in mice (Hendrickson et al. 2011; Liu et al. 2012). Additionally, mice carrying a knockout mutation in the $\alpha 4$ nAChRs were reported to consume significantly less ethanol than wild-type mice in the Drinking in the Dark (DID) paradigm (Hendrickson et al. 2011). Furthermore, the development of cross-tolerance between nicotine and ethanol has also been observed (Collins et al. 1988) in mice.

The initial response to a drug is likely to be one factor contributing to the risk for future abuse or dependence (de Wit and Phillips 2012). Indeed, work by Schuckit et al. (1987) and others (Eng et al., 2005) have strongly suggested that a low level of intoxication-like response to ethanol in non-dependent individuals is a significant risk factor for future ethanol dependence. Individuals that are relatively insensitive to the effects of ethanol (low level of response) are relatively more susceptible to developing alcohol use disorders. In contrast, increased sensitivity to the unpleasant subjective effects of intoxication, such as ataxia and sedation, has been posited to serve as a protective influence by discouraging drinking (Krystal et al. 2003). Similarly, higher ethanol consuming lines of mice are less sensitive to the sedative-hypnotic and ataxic effects of ethanol (Shen et al. 1996; Phillips et al. 2002). Preclinical data have suggested that nicotine may initially reduce the aversive effects of ethanol. For example, nicotine reverses ethanol-induced cognitive impairments and conditioned taste aversion in rodents (Kunin et al. 1999; Gulick and Gould 2008). In addition, an intracerebellar nicotine injection attenuates ethanol-induced ataxia in mice (Al-

Rejaie and Dar 2006) via $\alpha 4\beta 2^*$ (* indicates additional subunits within the nicotinic acetylcholine receptor complex) and $\alpha 7$ nAChR subtypes (Taslim et al. 2008, 2011). We therefore hypothesized that nicotine would decrease the duration of the loss of righting reflex (LORR), a common measure of ethanol's sedative-hypnotic effects.

The purpose of the present experiments was to examine the effects of nicotine administration on the duration of ethanol-induced LORR and to characterize their pharmacological interactions in mice through dose-response, receptor efficacy, strain difference and tolerance measures. We also evaluated the involvement of major nAChR subtypes in mediating the effects of nicotine on ethanol sedation. To understand if the effect of nicotine on ethanol extended to other behaviors, we investigated the impact of nicotine on ethanol intake in the DID paradigm and ethanol-induced hypothermia as well.

Materials and Methods

Animals

Male C57BL/6J and DBA/2J mice were purchased from Jackson laboratories (Bar Harbor, ME). Mice carrying null mutations in $\alpha 7$ (*Chrna7*; Jackson Laboratories) and $\alpha 4$ (*Chrna4*) nAChR subunits (provided by Dr. Henry Lester at the California Institute of Technology, with the permission of Dr. John Drago) (Ross et al. 2000) and their wild-type (WT) littermates were bred in the animal care facility at Virginia Commonwealth University. Each mutant strain was backcrossed 10 – 12 times against the parental strain (C57BL/6J) before being used. Mutant strains were maintained as heterozygotes; experimental animals were obtained from crossing heterozygote mice to generate mutant and wild-type littermates. All experiments were performed on male mice. Mice were housed in a 21°C humidity-controlled Association for Assessment and Accreditation of Laboratory Animal Care (AALAC)-approved animal care facility. They were housed in groups of six and had free access to food and water under a 12-h light/dark cycle (lights on at 7:00 a.m.) schedule. Mice were 8–10 weeks of age and weighed approximately 25–30 g at the start of each experiment. All experiments were performed during the normal light cycle (between 7:00 a.m. and 7:00 p.m.) and the study was approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University. All studies were carried out in accordance with the National Institute of Health guide for the Care and Use of Laboratory animals.

Drugs

(-)-Nicotine hydrogen tartrate salt [(-)-1-methyl-2-(3-pyridyl) pyrrolidine (-)-bitartrate salt] and mecamylamine were purchased from Sigma-RBI (Natick, MA, USA). Ketamine HCl was purchased from Vedco Inc. (Saint Joseph, MO). Varenicline and sazetidine were obtained from the Drug Supply Program of the National Institute on Drug Abuse (Rockville, MD). These drugs were dissolved in 0.9% saline and injected subcutaneously (s.c.) at a volume of 10 ml/kg body weight. Ethanol was also dissolved in 0.9% saline and prepared as a 20% (v/v) solution and was delivered via intraperitoneal (i.p.) injection. All doses are expressed as the free base of the drug.

Loss of Righting Reflex (LORR) studies

Ethanol and Nicotine Dose Response Curves—The sedative-hypnotic effects of ethanol were measured using the loss of righting reflex (LORR) assay. We generated an ethanol dose response curve by injecting mice with varying doses of ethanol (2.5, 3.0, 3.5, 4.0 g/kg) (Crabbe et al., 1994). In establishing a dose response curve for nicotine plus ethanol, mice were injected with varying doses of nicotine (0.05, 0.1, 0.5, 1.0 mg/kg, s.c.) (Jackson et al., 2009) and 5 min later received 2.5g/kg of ethanol i.p. The assay started immediately after the ethanol injection and mice were monitored for initial LORR; mice were placed in a supine position in a V-shaped trough, and the time at which mice were unable to right themselves from a supine position was recorded. A subject was confirmed to have achieved LORR only after it was on its back for at least 30 sec. We measured the total time required for the subject to right itself 3 times within 30 sec from the onset of LORR, which was reported as LORR Duration. Data (mean $\pm$ S.E.M.) were expressed as LORR duration in min.

Dose-Addition Analysis—Drug interactions were assessed using both graphical and statistical approaches to dose-addition analysis (Tallarida 2006) as described previously (Banks et al. 2010). Graphically, data for each drug and drug mixture were plotted as isobolograms at the effect level that produced a LORR of 2886 sec, which represents an approximate 50% maximal effect level. Thus, these isobolograms plotted ethanol dose $\pm$ S.E.M. in a mixture as a function of nicotine dose $\pm$ S.E.M. in the mixture at the overall mixture dose that produced a LORR of 2886 sec. Statistical evaluation of drug interactions was accomplished by comparing the experimentally determined ED₅₀ values for each mixture (Z_{mix}) with predicted additivity ED₅₀ values (Z_{add}) as described by Tallarida (2000). Z_{mix} values were determined empirically by either interpolation or linear regression of group dose effect functions. Z_{add} values were calculated based on the additivity hypothesis that predicts the inactive drug (nicotine) should not contribute to the effects of a mixture. As a result, the equation for Z_{add} reduces to $Z_{\text{add}} = A/\rho A$, where A was the ED₅₀ for ethanol alone, and ρA is related to the proportion of ethanol in a mixture according to the equation $\rho A = fA/Z_{\text{add}}$. Mean Z_{mix} and Z_{add} values were considered significantly different if 95% confidence limits did not overlap.

Importance of Receptor Efficacy—For that varenicline and sazetidine, partial agonists at $\alpha 4\beta 2^*$ nAChRs, were used. Mice were injected with varenicline (0.1, 1 and 4 mg/kg, s.c.) or sazetidine (0.1, 1 and 3 mg m/kg, s.c.) and 5 min later they received 2.5g/kg of ethanol. LORR duration was then recorded.

Determining the Role and Subtypes of Nicotinic Receptors—We first used mecamylamine, a nonselective nicotinic antagonist. Mice were injected with either saline or mecamylamine (2.0mg/kg, s.c.). Ten min later, mice were injected with either saline or 0.5 mg/kg of nicotine, and then 5 min later, they were injected with 2.5 g/kg of ethanol. LORR duration was then recorded.

The role of $\alpha 4^*$ and $\alpha 7$ nAChR subtypes was assessed using their respective WT and KO mice. Both WT and KO mice were injected with either saline or 1.0mg/kg nicotine and 5 min later, injected with 2.5g/kg ethanol. LORR duration was then recorded.

Ketamine and Pentobarbital-Induced Loss of Righting Reflex—LORR was used to study the hypnotic effects of ketamine. Male C57BL/6J mice were administered a s.c. injection of either saline (n=8) or 1 mg/kg nicotine (n=8). 5 min after the initial injection, mice received an i.p. injection of 100 mg/kg ketamine HCl or 30 mg/kg of pentobarbital and the latency to LORR was recorded.

Repeated Nicotine Exposure—Male C57BL/6J mice were injected twice a day (8-hrs apart) with either saline or 2.0 mg/kg nicotine for 4 consecutive days. On Day 5, mice were injected with 1.0 mg/kg nicotine and then 2.5 g/kg ethanol 5 min later. LORR duration was recorded. This repeated nicotine protocol was previously shown to induce tolerance to several acute effects of nicotine in the mouse (Damaj and Martin, 1996).

Strain Differences—We compared the effect of nicotine on ethanol-induced LORR in ethanol-preferring C57BL/6J versus ethanol non-preferring DBA/2J mice. C57BL/6J and DBA/2J mice were injected with saline, 0.5 or 1.0 mg/kg nicotine and then 2.5 g/kg ethanol 5 minutes later. LORR duration was recorded. The effect of nicotine (1 mg/kg, s.c.) on blood ethanol concentrations (BEC) was determined in both strains as described below.

The effect of nicotine on BEC in C57BL/6J and DBA/2J mice

C57BL/6J and DBA/2J mice were injected with either saline or 1.0mg/kg nicotine and 5 min later injected with 2.5g/kg ethanol. Blood samples were taken from the cheek and analyzed 15, 60, and 180 min following ethanol injection. Whole blood samples (20 μ l) were placed into 20-ml headspace vials with 960 μ l water and 20 μ l 1-propanol internal standard. Samples were tested for ethanol concentration using a Hewlett Packard 5890A gas chromatograph equipped with a flame ionization detector, 2 meter 5% Carbowax 20M 80/120 mesh packed column (Restek, Bellefonte, PA) and CTC Combi-Pal headspace autosampler.

Nicotine blood levels in C57BL/6J and DBA/2J mice

C57BL/6J and DBA/2J mice were injected with 1.0mg/kg nicotine and 15 min later, trunk blood was collected following decapitation and was immediately centrifuged for 10 minutes. Blood plasma was stored for 7 days at -80°C . Plasma nicotine levels were measured using high-performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS) analysis as previously described (AlSharari et al., 2013). At least five animals were used per group.

Body Temperature Measurement

Hypothermia induced by acute ethanol was measured using a standard rectal thermometer (Fischer Scientific, Pittsburg, PA) with probe (inserted ~ 24 mm). After baseline temperatures were recorded, B6 mice were injected with nicotine (0.05, 0.1, 0.5 mg/kg) or saline 5 min before treatment with 2.5 g/kg ethanol or saline. Body temperature was

recorded at 15 min after ethanol injection. Data were expressed as mean $\pm$ SEM of the change in body temperature from baseline after treatment. The ambient temperature of the laboratory varied from 21–24°C from day to day.

Drinking-in-the-Dark (DID)

DID is a limited access drinking procedure used to model sub-chronic binge-drinking behavior in rodents (Hendrickson et al. 2009). B6 adult male mice were single-housed one week prior to testing with *ad libitum* access to food and water. As mice have been shown to display maximal ethanol consumption a few hours into the dark cycle (Rhodes et al. 2005), housing in a reverse light-dark cycle (7:00 am – 7:00 pm) facilitated daytime testing. At the end of the acclimation period, three hours into the dark cycle (10:00 am), the water bottle from each cage was replaced with a drinking tube containing 20% (w/v) ethanol. Baseline ethanol intake was measured for two days at four hours after ethanol presentation.

In a separate group of B6 mice who were singly-housed one week prior to testing with *ad libitum* access to food and water, we investigated the impact of acute vehicle (saline) or nicotine (0.1 or 0.5 mg/kg, s.c.) injection on ethanol intake in the DID procedure. All mice were habituated with saline injections for three days (once a day). On the fourth day, each group was treated with either vehicle (saline) or nicotine immediately before presenting ethanol and all volume measurements were taken at four hours after the presenting ethanol. Ethanol consumption data (mean $\pm$ SEM) were expressed as total intake in g/kg.

Statistical Analysis

Data in the LORR test were analyzed using analysis of variance (ANOVA) with treatment, and/or genotype as independent variables. Data for the DID drinking assay and hypothermia were analyzed using one-way ANOVA. All analyses were followed by Bonferroni post-hoc tests, where appropriate, to further analyze significant data with the null hypothesis rejected at an alpha level of 0.05.

Results

Nicotine dose-dependently enhances ethanol-induced LORR

We tested the acute effect of a range of ethanol doses and found that ethanol produces dose-dependent increase in the duration of sleep in the LORR assay in B6 mice (Fig. 1A). 2.5 g/kg produced a brief sleeping period of 27 min and all higher doses induced a significantly longer duration than 2.5 g/kg (one-way ANOVA, [$F_{(3,26)} = 63.4$; $p < 0.0001$]). We used the lowest active dose of ethanol (2.5 g/kg) to investigate nicotine's effect on ethanol-induced LORR. We pretreated the animals with vehicle or nicotine, after which mice were injected with 2.5g/kg i.p. ethanol. Doses of 0.1 mg/kg nicotine and higher significantly increased LORR duration relative to ethanol injection alone one-way ANOVA, [$F_{(3,22)} = 28.6$; $p < 0.0001$] in a dose-dependent manner (Fig. 1B). At the highest dose of 1 mg/kg, nicotine increased the duration of ethanol-induced LORR 9-fold (Table 1). In addition, nicotine pretreatment (0.5 mg/kg, s.c.) potentiated the hypnotic effect of ethanol as is evidenced by a leftward shift in ethanol's dose-response curve (Fig. 1C). Nicotine when injected alone and up to a dose of 2 mg/kg did not cause any LORR or sleep in mice.

We next examined the nature of nicotine-ethanol on the duration of sleep in B6 mice. The predicted Z_{add} values and the empirically determined Z_{mix} values for the 0.2:1 Nicotine/Ethanol mixture are 0.59 (0.54–0.65) and 0.44 (0.38–0.50) respectively. Figure 1D shows the corresponding isobologram for the interaction. The 0.2:1 nicotine/ethanol mixture produced a synergistic LORR effect as indicated by the empirically determined Z_{mix} value being significantly lower than the predicted Z_{add} value. Graphically, this nicotine/ethanol mixture point was located to the left of the ethanol alone point in the isobologram. Furthermore, the ED_{50} value ($\pm$ s.e.m.) for ethanol alone to produce LORR was 3.35 ($\pm$ 0.07) compared to the ED_{50} value of ethanol in combination with nicotine 1.98 ($\pm$ 0.14).

Varenicline and sazetidine enhance the hypnotic effects of ethanol

Varenicline increased ethanol-induced LORR (Fig. 2A). The highest dose of 4.0mg/kg varenicline significantly increased LORR duration greater than after ethanol injection alone (2.5-fold increase, Table 1), one-way ANOVA, [$F_{(2,21)} = 6.5$; $p < 0.001$]. Similarly, sazetidine, potentiates ethanol's hypnotic effect at doses 1.0mg/kg or 3.0 mg/kg [$F_{(3,19)} = 103.03$; $p < 0.0001$] (Fig. 2B). Varenicline and sazetidine given at the doses used in this study did not produce LORR on their own [Duration of LORR = 0 ± 0 min at 1 and 4.0 mg/kg] [Duration of LORR = 0 ± 0 min at 1 and 3.0 mg/kg]. Varenicline was found to be less efficacious than nicotine and sazetidine in increasing LORR duration (Table 1).

Mecamylamine blocks the enhancement by nicotine of ethanol-induced hypnosis

A dose of 2.0 mg/kg mecamylamine blocked nicotine's enhancement on ethanol's hypnotic effect (Fig. 3) (one-way ANOVA, [$F_{(3,28)} = 13.8$; $p < 0.0001$]). Mecamylamine did not significantly alter ethanol-induced LORR ($p > 0.05$) on its own.

$\alpha 4^*$ and $\alpha 7$ nAChR subtypes are required for nicotine's effect on ethanol-induced LORR

In order to determine which nAChR subtypes mediate the interaction between ethanol and nicotine, we used mice that were carrying null mutations for either the $\alpha 4$ or $\alpha 7$ nicotinic subunits, and tested them for the ability of nicotine to enhance ethanol-induced LORR. $\alpha 4$ KO mice and their WT littermates were pretreated with nicotine (1 mg/kg, s.c.) and 5 min later they were injected with 2.5g/kg i.p. ethanol. We found that $\alpha 4$ KO mice pretreated with nicotine did not show a significant enhancement of ethanol's hypnotic effect as compared to WT mice (one-way ANOVA, [$F_{(5,40)} = 48.4$; $p < 0.0001$], Fig. 4A), indicating that the $\alpha 4$ -containing nAChR is required for this effect of nicotine. In addition, $\alpha 7$ receptor KO mice demonstrated a smaller nicotine-induced enhancement of LORR duration compared to WT mice (25% decrease) (one-way ANOVA, [$F_{(3,20)} = 18.8$; $p < 0.0001$], Fig. 4B). However, nicotine's enhancement of ethanol LORR was still significant in $\alpha 7$ KO mice compared to saline-treated animals.

Nicotine-ethanol interaction in C57BL/6J and DBA/2J mice

We tested if we could detect strain differences for the nicotine-ethanol interaction in the LORR test. C57BL/6J and DBA/2J mice were pretreated with nicotine (0.5 and 1 mg/kg, s.c.) or saline and 5 min later they were injected with 2.5g/kg i.p. ethanol. Nicotine pretreatment significantly enhanced ethanol LORR duration in both C57BL/6J (Fig. 5A) and

DBA/2J mice (Fig. 5B). Intriguingly, nicotine was less potent (approximately 50% less potent) in enhancing ethanol LORR duration in DBA/2J compared to C57BL/6J mice [$F_{(5,35)} = 84.1$; $p < 0.0001$] (Fig. 5B and Table 2).

C57BL/6J and DBA/2J mice have similar blood ethanol concentrations

In order to rule out metabolic differences in the effects of nicotine between the two strains, we analyzed blood samples of C57BL/6J and DBA/2J mice pretreated with vehicle or 1.0mg/kg nicotine. 5 min after pretreatment, mice were injected with 2.5g/kg i.p. ethanol. Samples were taken at three time points (15, 60, and 180 min) post-injection. No significant differences in BEC between mice receiving vehicle and mice receiving nicotine in both B6 (Fig. 6A) and DBA/2J mice were found (Fig. 6B).

C57BL/6J and DBA/2J mice have similar blood nicotine levels

To determine if the differences in the potency of the nicotine effect on ethanol LORR observed between C57BL/6J and DBA/2J mice were not due to a different tissue level of nicotine, we determined the nicotine levels in the blood of these mice treated with nicotine at the dose of 1 mg/kg (C57BL/6J: 91 ± 7 ng/ml; DBA/2J: 85 ± 12 ng/ml). No differences in the plasma nicotine levels in these strains were found, which is consistent with results reported by Siu and Tyndale (2007) in the same strains.

Nicotine induced augmentation of ketamine and pentobarbital in mice

We investigated if nicotine could alter the effects of other sedative drugs. As ethanol has been shown to generally positively modulate γ -aminobutyric acid A receptor (GABA_A) and inhibit N-Methyl-D-aspartate (NMDA) receptors, we tested the effects of nicotine on ketamine- and pentobarbital-induced LORR (Harris et al. 2008). As seen in Figure 7, nicotine at 1 mg/kg enhanced both ketamine (100 mg/kg) and pentobarbital (30 mg/kg) sedative effects ($p < 0.05$).

Chronic tolerance develops to the enhancement of ethanol's effects by nicotine

We then determined if repeated nicotine produces tolerance to its enhancement of the hypnotic effect of ethanol. Mice were injected with nicotine or saline (1 mg/kg bid for 4 days) and then on day 5, mice were challenged with 1.0mg/kg nicotine and ethanol. As shown in Figure 8, we observed a significant partial reduction of nicotine-induced enhancement of ethanol LORR in mice given repeated nicotine [$F_{(4,40)} = 33.75$; $p < 0.0001$] compared to those treated with saline.

Nicotine enhances ethanol-induced hypothermia

We tested if nicotine also enhances ethanol's hypothermic effects. Mice received an injection of saline or nicotine and 5 min later a dose of 2.5 g/kg ethanol (i.p.). Fifteen min post ethanol injection, mice body temperature was measured. One-way ANOVA revealed there is a significantly greater hypothermic enhancement of ethanol by nicotine in mice that received 0.05, 0.1, or 0.5 mg/kg compared to the saline treated mice [$F_{(5,32)} = 47.22$, $p < 0.0001$] (Figure 9A).

Effect of acute nicotine on ethanol intake in the DID test

Finally, we assessed the impact of acute nicotine at doses that were shown to impact ethanol-induced LORR (0.1 and 0.5 mg/kg, s.c.) on ethanol consumption in B6 male mice using the DID paradigm. As intended, saline-treated B6 mice drank ethanol with blood levels sufficient to produce behavioral intoxication ($\text{BEC} > 1.0 \text{ mg/ml}$ - Data not shown) after 4-hrs of ethanol exposure on the day of testing (Figure 9B). Acute nicotine injection attenuated ethanol intake in mice (Figure 9B) [$F(2, 26) = 20.00$; $p = 0.001$] with post hoc analysis showing significance at the dose of 0.5 mg/kg of nicotine ($p < 0.05$) with a lower ethanol intake in nicotine-treated compared to saline-treated mice.

Discussion

The pharmacological interaction between nicotine and ethanol could play a role in their common co-abuse. The goal of our studies was to investigate the pharmacological impact of nicotine on the physiological effects of ethanol in a mouse model. We found that nicotine synergistically enhances ethanol-induced LORR, and that this enhancement is mediated by $\alpha 4^*$ and $\alpha 7$ nAChRs. In addition, this pharmacological interaction is modulated by genetic differences between mouse lines. Nicotine also enhanced the hypothermic response to ethanol but caused a reduction in ethanol intake in the DID paradigm.

Nicotine dose-dependently enhanced the duration of sleeping time at the lowest LORR-inducing ethanol dose (2.5g/kg), and this interaction between ethanol and nicotine was synergistic. While the precise mechanisms of this interaction were not investigated in our study, the enhancement of LORR by nicotine was blocked by mecamylamine, a non-selective nAChR antagonist, demonstrating that these effects of nicotine are mediated by nAChR activation. We found that $\alpha 4$ KO mice lacked nicotine enhancement of the duration of ethanol-induced LORR compared to their WT littermates, strongly supporting a functional role for $\alpha 4$ -containing nAChR subtypes in this interaction. Interestingly, the $\alpha 6$ nicotinic subunit, which can form a functional receptor with $\alpha 4$ and $\beta 2$ subunits, was previously reported to play a role in ethanol-induced LORR. Indeed, $\alpha 6$ KO mice showed an enhancement in the duration of LORR compared to their WT counterparts (Kamens et al. 2012). Our data with $\alpha 4$ KO mice suggests that $\alpha 6\beta 2^*$ but not the $\alpha 6\alpha 4\beta 2^*$ subtypes may mediate the effect of $\alpha 6$ subunit in the LORR response. In addition, $\alpha 7$ KO mice showed a significant but partial reduction in nicotine's effect on LORR compared to WT mice. This could imply that the $\alpha 7$ nicotinic subtype plays a modulatory role in nicotine's effects on the LORR. An earlier study reported that $\alpha 7$ KO mice are more sensitive to ethanol's hypnotic effects in the LORR test when the drug was given at 3.8 g/kg (Bowers et al. 2005); however in our studies, we did not observe a difference in the sensitivity to ethanol's hypnotic effects on $\alpha 7$ KO mice relative to WT at the dose of 2.5 g/kg (Figure 4b, saline-treated).

We also tested whether the LORR-enhancing effect of nicotine was specific to ethanol. As ethanol has been shown to act prominently by positive modulation of GABA_A and inhibition of NMDA receptors (Grant et al. 1994), we tested whether nicotine also modulated pentobarbital- and ketamine-induced LORR. Nicotine increased the duration of LORR induced by both ketamine and pentobarbital. Although the mechanisms of ethanol-induced LORR are not completely understood, these results suggest that molecular events

downstream of ethanol, ketamine or pentobarbital direct action might be a site for nicotine enhancement of LORR from all three drugs. For example, both ethanol- and GABA_A-agonist-induced LORR were inhibited in mice lacking adenylyl cyclase type 5 (Kim et al., 2012). Further study of nicotine enhancement of ethanol, ketamine and pentobarbital LORR might thus assist in identifying mechanisms of nicotine action on these sedative responses.

Consistent with a previous study (Kamens et al. 2010), we found that varenicline, a $\alpha 4\beta 2^*$ partial agonist, significantly increased the duration of ethanol-induced LORR. However, the magnitude of increase by varenicline was substantially less than that evoked by nicotine (9-fold difference at the dose of 1 mg/kg), suggesting that there is an important role for receptor efficacy in the nicotine-ethanol interaction. Surprisingly, sazetidine, a more selective $\alpha 4\beta 2^*$ partial agonist, was less efficacious than nicotine at low doses (0.1 mg/kg). However, it was more efficacious than nicotine at higher doses (1 mg/kg) in this test. While sazetidine is a highly selective $\alpha 4\beta 2$ receptor agonist (Xiao 2006), varenicline also acts as a full agonist at the $\alpha 3\beta 4^*$ and $\alpha 7$ nAChR subtypes (Mihalak et al. 2006). These results suggest that the additional nAChR subtypes may also play a role in the interaction between nicotine and ethanol; this is consistent with our observation on the $\alpha 7$ KO mouse. In addition, the $\alpha 4\beta 2$ receptor can exist in two forms: the high-affinity $\alpha 4(2)\beta 2(3)$ or the low-affinity $\alpha 4(3)\beta 2(2)$ (Moroni and Bermudez, 2006). Sazetidine acts as a full agonist on the high affinity $\alpha 4\beta 2$ subtype (Zwart et al. 2008) and this may be the primary subtype mediating its effects on LORR.

We found that mouse genotype plays an important role in the acute nicotine-ethanol interaction in the LORR test. DBA/2J mice were less sensitive than C57BL/6J to the enhancing effects of nicotine on ethanol-induced LORR. Even though the BEC in the DBA/2J mice was higher at time points 15 and 60 min compared to C57 mice in vehicle-treated groups, nicotine did not alter BEC in either strain. This strongly suggests that the interaction of nicotine with ethanol does not involve metabolic factors after acute administration. In addition, nicotine plasma levels time course did not differ between the two strains. Collectively, these observations argue for a pharmacodynamic difference between the two strains. While we observed no difference in the duration of ethanol-induced LORR between the strains in the absence of nicotine, the C57BL/6J and DBA/2J inbred strains are known to exhibit pronounced differences in several ethanol behaviors (Crabbe et al. 1994; Cunningham et al. 2014). For example, C57BL/6J mice self-administer more ethanol in the schedule-induced polydipsia paradigm (Mittleman et al. 2003) and show a preference for ethanol in the two-bottle choice test compared to DBA/2J mice (Crabbe et al. 1994). However, they are less sensitive to the rewarding effects of ethanol in the conditioned place preference test (Cunningham et al. 1992). These strains also differ in their nicotine responses (Jackson et al. 2009; 2011). DBA/2J mice are less sensitive to the acute effects of nicotine compared to C57BL/6J (Jackson et al. 2009). It is therefore possible that differences in nicotinic mechanisms and nAChR signaling between the C57BL/6J and DBA/2J mice mediate the differential sensitivity of nicotine-ethanol interaction in LORR.

As expected, tolerance to nicotine's enhancing effects on LORR developed after repeated administration. This agrees with a study that shows the production of chronic nicotine

tolerance for the interactive effects of ethanol and nicotine on learning in C57BL/6J mice (Gulick and Gould 2008).

In contrast to the enhancement by nicotine of the hypnotic and hypothermic responses to ethanol, nicotine and other nicotinic agonists have previously been shown to attenuate ethanol-induced ataxia measured in the rotarod test (Taslim et al. 2008, 2011) in mice. In addition, nicotine reverses ethanol-induced learning deficits (Gould and Lommock 2003; Gulick and Gould 2008; Rezayof et al. 2008; Tracy et al. 1999) in mice and rats. These results suggest that nicotine differentially modulates acute responses of ethanol in rodents.

Human and animal data have shown that an increased sensitivity to ethanol decreases drinking behavior. It is posited that the reason for this is that enhancing the unpleasant subjective effects of ethanol, such as sedation, serves as a protective influence and discourages consumption (Krystal et al. 2003; Shen et al. 1996; Phillips et al. 2002). Therefore, we predicted that acute nicotine treatment would decrease ethanol drinking. Indeed, using the DID paradigm, a model of binge drinking (Rhodes et al. 2005), we observed that acute nicotine significantly reduced ethanol intake, which is in agreement with previous studies on ethanol drinking (Hendrickson et al. 2009).

However, the effects of nicotine on ethanol intake are not simple. Rat studies show that acute nicotine exposure increases ethanol oral self-administration, reinstatement and drinking (Doyon et al. 2013; Le et al. 2003; Smith et al. 1999). In addition, bilateral nicotine injections into the basal forebrain increased ethanol consumption in the DID test in B6 mice (Sharma et al. 2014). In contrast, several additional mouse studies report that, consistent with our results, acute nicotine and nicotinic agonists decrease ethanol consumption in the DID and two-bottle choice tests (Hendrickson et al. 2009, 2011; Sajja and Rahman 2010). It is thus likely that there are species differences in the nicotine and ethanol interaction. Secondly, differences in the method of nicotine administration or assessment of ethanol consumption were different in these various studies (Operant ethanol self-administration versus choice or DID drinking protocols). Overall, our results highlight the need for further analysis to elucidate mechanisms mediating the behavioral interaction between nicotine and ethanol.

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ABBREVIATIONS

LORR	loss of righting reflex
nAChR(s)	nicotinic acetylcholine receptor(s)
s.c.	subcutaneous injection; subunits
i.p.	intraperitoneal injection
WT	wildtype

KO	knockout
CNS	central nervous system
DID	Drinking in the Dark

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Figure 1. Nicotine enhances ethanol's hypnotic effects in a synergistic manner

Data (mean $\pm$ SEM) represent (A) total duration of LORR in min in C57BL/6J mice after i.p. injection of ethanol [$*p < 0.0001$ vs 2.5g/kg EtOH] and (B) duration of LORR in mice after nicotine pretreatment at various doses after receiving 2.5g/kg ethanol [$*p < 0.0001$ vs saline, $\#p < 0.0001$ vs 1.0mg/kg nicotine]. N=6–12 per group. (C) A dose response curve showing duration of LORR in min after i.p. injection of ethanol (squares) and with nicotine pretreatment after i.p. injection of ethanol (circles) [$*p < 0.0001$ vs EtOH+Nic]. (D) Effects of ethanol alone and in combination with nicotine on LORR. Isobologram shows LORR at the ED50 effect level for ethanol alone, and ethanol and nicotine as part of a mixture. Ordinate: ED50 values for nicotine as part of a mixture in mg/kg (linear scale). Abscissae: ED50 values for ethanol alone or in a mixture in g/kg (linear scale). Each point represents mean $\pm$ s.e.m. of 30 mice. Asterisk indicates the nicotine:ethanol mixture produced a synergistic effect as determined by dose-addition analysis.

Figure 2. Receptor efficacy plays a role in the interaction between nicotine and ethanol
Data (mean $\pm$ SEM) represent total duration of LORR in min in C57BL/6J mice with (A) varenicline pretreatment and (B) sazetidine pretreatment after receiving an injection of 2.5g/kg ethanol [$*p < 0.006$ vs saline]. N=6–10 mice per group.

Figure 3. Mecamylamine blocks nicotine's enhancement of ethanol's hypnotic effect

Data (mean $\pm$ SEM) represent (A) duration of LORR in min in C57BL/6J mice after mecamylamine and nicotine pretreatment before receiving an injection of 2.5g/kg ethanol [$*p < 0.0001$ vs ethanol, $\#p < 0.002$ vs Nicotine+EtOH]. N=10–11 mice per group.

Figure 4. $\alpha 4$ and $\alpha 7$ nicotinic acetylcholine receptor subunits may play a role in nicotine/ethanol interaction

Data (mean $\pm$ SEM) represent duration of LORR in min in (A)- $\alpha 4$ KO and WT and (B)- $\alpha 7$ KO and WT mice receiving nicotine (1 mg/kg, s.c.) pretreatment followed by an injection of 2.5g/kg ethanol. [*p<0.009 vs saline, #p<0.0001 vs WT nicotine]. N=6 mice per group.

Figure 5. Strain differences observed in the enhancement of ethanol's hypnotic effects by nicotine among C57BL/6J (open bars) and DBA/2J (solid bars) mice

Data (mean $\pm$ SEM) represent duration of LORR in (A) C57BL/6J [$*p < 0.0001$ vs saline] and (B) DBA/2J [$*p < 0.0001$ vs saline, $\#p < 0.0001$ vs C57BL/6J nicotine] mice with nicotine pretreatment after receiving an injection of 2.5g/kg ethanol. N=6 mice per group.

Figure 6. Nicotine does not affect ethanol plasma levels in C57BL/6J and DBA/2J mice
Strains do not show a difference in ethanol metabolism when given a 5min pretreatment with vehicle or 1.0mg/kg nicotine and then receiving an injection of 2.5g/kg ethanol. (A) C57BL/6J and (B) DBA/2J mice blood samples were taken at 15, 60, and 180 min post-ethanol injection. Blood ethanol concentration (BEC) is reported as mg/mL. N=5 mice per group.

Figure 7. Nicotine enhances ketamine and pentobarbital's hypnotic effects in mice
Data (mean $\pm$ SEM) represent duration of LORR in mice with nicotine pretreatment (1 mg/kg, s.c.) after receiving an injection of (A) ketamine (100 mg/kg, i.p.) or (B) pentobarbital (30 mg/kg, i.p.). N=6–8 mice per group.

Figure 8. Tolerance developed to the enhancement of ethanol's hypnotic effects by nicotine
 Data (mean $\pm$ SEM) in the right side (under Chronic) represent total duration of LORR in C57BL/6J mice with repeated injections of saline (first two columns) or nicotine (2 mg/kg, s.c.) (third column) for 4 days. On Day 5, mice received a 5 min saline or nicotine pretreatment (1 mg/kg, s.c.) before receiving an injection of 2.5g/kg i.p. ethanol. Data in the left side (under Acute) represents the effects of an acute dose of nicotine (1 mg/kg, s.c.) 5 min before an injection of 2.5g/kg i.p. ethanol in a separate group of animals [$*p < 0.0001$ vs Saline, $\#p < 0.0001$ vs Saline+Nicotine]. $N = 7-8$ mice per group. Nic = Nicotine.

Figure 9. Effects of acute nicotine on other behavioral responses to ethanol

Nicotine enhances ethanol-induced hypothermia in mice. (A) Changes in body temperature in mice with nicotine pretreatment at various doses after receiving an injection of 2.5g/kg ethanol [$*p < 0.05$ vs saline, $\#p < 0.05$ vs 2.5 g/kg ethanol]. N=6–8 mice per group. (B) Nicotine injection (saline, 0.1 and 0.5 mg/kg, s.c.) treatment decreased EtOH intake consumption in B6 mice in the DID paradigm. Data (mean + SEM) represent daily DID

ethanol intake in g/kg for 4 hours (N= 8–10/group). * $p < 0.05$ compared to saline-treated group. N=7–8 mice per group.

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Table 1

Varenicline is less efficacious than nicotine and sazetidine in increasing LORR duration induced by ethanol.

Dose (mg/kg)	Nicotine	Varenicline	Sazetidine
0.1	4.2 ± 0.9 [*]	0.4 ± 0.1	1.4 ± 0.6
0.5	4.9 ± 1.2 [*]	NT [#]	NT [#]
1.0	9.2 ± 0.7 [*]	1.0 ± 0.2	11.1 ± 1.0 [*]
3.0	NT [#]	NT [#]	14.0 ± 0.1 [*]
4.0	NT [#]	2.4 ± 0.5 [*]	NT [#]

Data (mean ± SEM) represent the magnitude (calculated as ratio of LORR duration of saline-treated group/drug-treated group) of increase in LORR duration in C57BL/6J mice induced by ethanol (2.5 g/kg) after pretreatment with various doses of nicotine, varenicline and sazetidine from Figure 1B.

^{*} p<0.05 vs saline-treated mice.

[#] NT= Not Tested.

Table 2

Nicotine enhances ethanol's hypnotic effects to a significantly greater magnitude in C57BL/6J mice compared to DBA/2J mice.

Nicotine (mg/kg)	C57BL/6J	DBA/2J
0.5	6.5 ± 0.6	3.2 ± 0.6 *
1.0	9.4 ± 0.7	4.7 ± 0.4 *

Data (mean ± SEM) represent the magnitude (calculated as ratio of LORR duration of saline group/nicotine group) of increase in LORR duration at two doses of nicotine in mice treated with ethanol at 2.5 g/kg.

*
p<0.05 vs C57BL/6J mice at corresponding nicotine dose.