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Nicotinic receptor ligands reduce ethanol intake by high alcohol-drinking HAD-2 rats

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Abstract

Neuronal nicotinic acetylcholine receptors (nAChRs) are implicated in the reinforcing effects of many drugs of abuse, including ethanol. The present study examined the efficacy of cytisine, a nAChR partial agonist, and lobeline, a putative nAChR antagonist, on the maintenance of ethanol drinking by HAD-2 rats. Adult male HAD-2 rats were given access to ethanol (15% and 30%, with ad lib water and food) 22 hr per day for 12 weeks, beginning at 60 days old, after which cytisine (0.0, 0.5 and 1.5 mg/kg) was tested for 3 consecutive days. The rats were given an 18 day wash-out period, and were then tested with lobeline (0.0, 1.0 and 5.0 mg/kg) for 3 consecutive days. Ethanol intake was measured at 1, 4 and 22 hours post-injection. Rats were injected i.p. just prior to lights out (1200 h). There was a significant main effect of cytisine treatment on the 2nd test day, with the 1.5 mg/kg dose significantly reducing ethanol intake at the 1 hr and 4 hr time-points, relative to saline, and the 0.5 mg/kg dose inducing a significant reduction at the 4 hr time-point. Conversely, lobeline treatment resulted in significant main effects of treatment for all 3 time points, within each test day, with the 5.0 mg/kg dose significantly reducing ethanol intake, relative to saline, at each time-point within each test day. These findings provide further evidence that activity at the nAChR influences ethanol intake and is a promising target for pharmacotherapy development for the treatment of alcohol dependence and relapse.

Keywords

Drug Addiction; Nicotinic receptor; Alcohol consumption; High-alcohol-consuming rats; Selectively bred rats; Cytisine; Lobeline

Introduction

Alcohol abuse and dependence remain public health problems, with the worldwide prevalence of alcohol use disorders being 1.7% and the prevalence in the United States being 8.5% with

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an annual cost of 185 billion dollars [National Institute on Alcohol Abuse and Alcoholism (NIAAA), 2007]. Whereas previous estimates of the ratio of men to women having an alcohol use disorder has varied between 1:2 and 1:3 (Brienza & Stein, 2002), more recent data suggests the “gender gap” has been narrowing in younger and older populations (Brienza & Stein, 2002; Nelson et al., 1998; Wilsnack et al., 1991). The deleterious effects of alcohol abuse on societal health are staggering (c.f., Brienza & Stein, 2002; Room et al., 2005). For example, the mortality of women with substance-associated diseases is four times that of breast cancer alone (Blumenthal, 1997), and a causal relationship has been shown between alcohol use and at least 50 different medical conditions (Rehm et al., 2003).

The central cholinergic system has been implicated in the development of alcohol and/or drug abuse (c.f., Larsson & Engel, 2004; Narahashi et al., 2001; Ribeiro-Carvalho et al., 2008; Rahman et al., 2008; Soderpalm et al., 2000). For example, there is ample evidence that both ethanol and nicotine increase extracellular dopamine (DA) in the nucleus accumbens (Tizabi et al., 2007) and particular subunits for the nicotinic acetylcholine receptor (nAChR: e.g., alpha-6 and beta-4) are implicated in ethanol’s stimulating effects (Kamens & Phillips, 2008). One possible mechanism for this effect is through activity at nAChRs in the ventral tegmental area (VTA: Blomqvist et al., 1997), particularly those expressing alpha-4 and beta-2 subunits (Olausson et al., 2007). Additionally, there is some evidence for a preferential role of anterior nucleus accumbens (Acb) nAChRs mediating ethanol’s effects on Acb-DA (Ericson et al., 2008). Moreover, nicotine pretreatment alters ethanol-induced DA release in the Acb shell (Lopez-Moreno et al., 2008). Genetically, the alpha3, alpha5, alpha7 and beta4 subunits of the nAChR have been implicated in a predisposition for ethanol stimulation and/or adolescent use of ethanol and tobacco (e.g., Greenbaum et al., 2006; Kamens et al., 2008; Rigbi et al., 2008; Schlaepfer et al., 2008). Pharmacologically, ligands for nAChRs with the alpha3, alpha6, beta2 or beta3 subunits alter ethanol self-administration and ethanol-induced increases in extracellular DA in the Acb (Kuzmin et al., 2008; Lof et al., 2007).

Animal models have been successfully used in developing treatments for a number of medical and psychiatric disorders (e.g., Griffin, 2002; McKinney, 2001). The selectively bred high alcohol-drinking (HAD-2) line of rat meets most of the proposed criteria (Cicero, 1979; Lester & Freed, 1973; McBride & Li, 1998), for a valid animal model of alcoholism (Bell et al., 2005; McBride & Li, 1998; Murphy et al., 2002). These animals will (a), as peri-adolescents (Bell et al., 2004) or adults (Bell et al., 2008), freely self-administer approximately 8 g/kg/day, or greater amounts, of ethanol under home-cage free-choice access conditions, when multiple concentrations of ethanol are made available; (b) achieve blood alcohol concentrations approximating 70 mg% (~ 2.3 g/kg/1 hr), after short deprivation periods, and 150 mg% (~ 6 g/kg/2 hr), after longer deprivation periods, under home-cage, free-choice self-administration conditions (Bell et al., 2008; Rodd et al., 2008, respectively); (c) will self-administer ethanol under operant conditions (Files et al., 1998; Oster et al., 2006; Samson et al., 1998); and (d) will display an alcohol deprivation effect [ADE, a model of relapse-like drinking (Bell et al., 2005; Rodd et al., 2004; Sinclair and Senter, 1967; Sinclair et al., 1973)] after repeated extended deprivation cycles under home-cage (Rodd et al., 2008; Rodd-Henricks et al., 2000) and operant self-administration conditions (Oster et al., 2006). Thus, the HAD-2 rat line, as an animal model of alcoholism, provides an opportunity to assess the efficacy of different pharmacological agents in reducing excessive ethanol consumption.

In the present study, we examined the effects of two nAChR ligands, cytisine and lobeline, on the maintenance of home-cage, free-choice ethanol self-administration by HAD-2 rats. Cytisine is a plant alkaloid with a relatively rigid conformation (Mihalak et al., 2006). In binding assays, cytisine is found to be selective for the alpha4beta2 nAChR subunit combination, compared with other important nAChR subtypes such as the alpha-3beta-4 and alpha-7 (Parker et al., 1998; Stauderman et al., 1998; Xiao et al., 2004). Furthermore, cytisine

shows greater potency at $\alpha 4\beta 2$ nAChRs compared with many other subunit combinations in functional assays (Chavez-Noriega et al., 2000; Slater et al., 2003). Cytisine is a high-efficacy agonist at $\alpha 7$ nAChRs and at various $\beta 4$ containing nAChRs, such as $\alpha 3\beta 4$; and functions as a low-efficacy partial agonist at $\alpha 4\beta 2$ and other $\beta 2$ containing nAChRs (Carbonnelle et al., 2003; Papke & Papke, 2002; Stauderman et al., 1998). Recently, cytisine has shown potential as a smoking cessation treatment (Tutka & Zatonski, 2005) and a derivative of cytisine has been found to inhibit ethanol intake and ethanol-seeking behavior (Coe et al., 2005; Steensland et al., 2007). However, the behavioral effects of cytisine on ethanol consumption in an animal model of alcoholism have not been investigated thus far.

Lobeline is a naturally occurring alkaloid obtained from the Asian plant, *lobelia inflata*. It is generally considered to be an agonist at nAChRs present in the central nervous system (Dwoskin & Crooks, 2002, see for review). Like cytisine, it shows promise as treatment for smoking cessation (Nunn-Thompson & Simon, 1989). However, lobeline has high affinity for nAChRs and acts as competitive and nonselective antagonist at $\alpha 4\beta 2$ and $\alpha 3\beta 2$ nAChRs (Teng et al., 1997; Dwoskin and Crooks, 2002). Furthermore, lobeline was found to inhibit the function of dopamine transporter (DAT) and vesicular monoamine transporter (VMAT) (Teng et al., 1997; Miller et al., 2004; Wilhelm et al., 2004). Lobeline inhibits the effect of psychostimulants in behavioral and neurochemical assays, such that lobeline reduces amphetamine-induced endogenous DA release from rat striatal slices (Miller et al., 2001). Lobeline was also found to inhibit nicotine-evoked [3H] DA overflow from rat striatal slices (Miller et al., 2000). Furthermore, lobeline has been shown to attenuate methamphetamine self-administration (Harrod et al., 2001). It is believed that the efficacy of lobeline to inhibit psychostimulant-induced effects is likely mediated by its activity at nAChRs and/or its ability to alter presynaptic dopamine storage and release (Miller et al., 2007). In addition to these effects, lobeline was found to function as μ opioid receptor antagonist, however it has less affinity than classical opioid receptor antagonist (Miller et al., 2007). Recently, lobeline has been shown to have some effect on alcohol preference in mice (Farook et al., 2009). Although the existing literature indicates that lobeline diminishes the behavioral and neurochemical effects of psychostimulants, as with cytisine, the effects of lobeline on ethanol consumption in an animal model of alcoholism have not been investigated thus far.

Materials and methods

Animals and ethanol drinking procedures

Animals used for this project were maintained in facilities fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Indiana University School of Medicine (Indianapolis, IN) and are in accordance with the guidelines of the Institutional Animal Care and Use Committee of the National Institute on Drug Abuse, National Institutes of Health, and the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, 1996).

Adult male HAD-2 rats ($n = 7/\text{dose}$), from the S50 and S51 generations, were obtained from the Indiana University School of Medicine (Indianapolis, IN) breeding colonies at 42 days of age and pair housed in plastic tubs, with wood chip bedding until late peri-adolescence (60 days old). The animal vivarium room was maintained on a reverse dark-light cycle (light offset at 1200h). At 60 days old, the rats were transferred to hanging stainless steel cages and had ad libitum access to food and water throughout the experiment. At 65 days old, the rats were given concurrent access to 15% and 30% ethanol (pharmaceutical grade 95% ethanol mixed in double-distilled water), and their body weight, water intake and ethanol intake monitored for 84 days. The animals had 24 hr access to their respective solutions except during the collection

of body and bottle weights each day. Starting on the 1st day of ethanol access, body weight, water bottle weight, and ethanol solution weights were obtained, to the nearest 0.1 g, with a Sartorius Balance BP 6100 and Sartorius Interface V24/V28-RS232C(-S)/423 (Sartorius Instruments, McGaw Park, IL) and recorded by a personal computer program (SoftwareWedge, Professional Edition v 5.0 for DOS; Sartorius Instruments) at least 5 days per week. Data, when missing (e.g., body weight and fluid volumes on some weekends), were taken as the average of values obtained from the nearest four previous and subsequent days. All weights were obtained at the same time each day, during the last 2 hr of the light cycle (1000–1200). Initially, the lower concentration [15% (vol./vol.)] was placed next to the water bottle away from the food hopper, with the higher concentration of ethanol [30% (vol./vol.)] placed farthest from the food hopper. Periodically the order of the ethanol concentrations was randomly changed. The water bottle was a standard glass bottle holding approximately 300 ml of fluid, with a stopper (no. 10) holding an angled (~135°) stainless steel sipper tube. Ethanol solutions were maintained in the same type glass bottles as the water solution, described above. Sipper tubes in the present study did not have a ball-bearing tip. Spillage was calculated by employing a “ghost-cage,” with the ethanol bottles and water bottle weighed daily, although a rat was not present. Approximately 0.5 ml of solution was spilled per weighing and this amount was subtracted from daily intake values for each respective solution. The ethanol bottles were refilled at least twice a week, and water bottles were refilled at least once a week. Bottles were replaced every 2 weeks.

Cytisine test procedures

At the end of the 100 days of ethanol access, the HAD-2 rats received daily injections of cytisine for 3 consecutive days. Ethanol and water intake were monitored prior to, during, and following the treatment period. The animals were randomly assigned to each dose group, with the average ethanol intake balanced across the cytisine dose (0.0, 0.5, and 1.5 mg/kg) groups. Similar doses of cytisine or its derivative have been used in ethanol drinking studies, in mice, previously (Hendrickson et al., 2009; Steensland et al., 2007). The dose of cytisine was mixed in sterile physiological saline (0.9%) and administered intraperitoneally 15-min before light offset each day. Ethanol and water bottles were removed 2 hr prior to light offset each day of the treatment week. During the 2 hr prior to light offset, body weight and water as well as ethanol bottle weights were obtained and recorded, as described above. Any other required husbandry issues (e.g., change-out of drip pan liners, changing bottles, etc.) were conducted during this 2 hr period as well. The respective animals' water and ethanol bottles were returned to the cages at light offset (1200h). Water and ethanol bottle weights were obtained at 1-hr, 4-hr and 22-hr after light offset.

Lobeline test procedures

Ethanol intake returned to basal levels within the 22-hr following the 3rd treatment of cytisine. Ethanol and water intake were monitored for an additional 2 weeks, with no differences in intake observed between the 3 original cytisine dose groups. Three weeks after the cytisine test week, the rats underwent treatment with lobeline for 3 consecutive days, with water, ethanol and body weights monitored as described during, and following, the cytisine test regimen described above. The 3 doses of lobeline were 0.0, 1.0 and 5.0 mg/kg, dissolved in sterile physiological saline (0.9%), and administered intraperitoneally 15-min before light offset. The doses of lobeline were selected based on a previous report examining its interactive effects with opioids (Miller et al., 2001). Three of the rats that received the lower dose of cytisine received the lower dose of lobeline, whereas the other 4 received the higher dose of lobeline. Three of the rats that received the higher dose of cytisine received the higher dose of lobeline, whereas the other 4 received the lower dose of lobeline. The saline control rats remained as saline controls for both nicotinic ligand tests.

Statistical analyses

Ethanol intake, ethanol percent preference [(volume of ethanol consumed/total fluid volume consumed)*100], water intake and body weight changes were assessed for each compound (cytisine and lobeline) separately. A one-way ANOVA was conducted for dose at each time point (1-, 4-, and 22-hr post-injection) within each day of treatment, which was followed by Dunnett t-test (2-tailed) planned comparisons between the dose and control (saline) group values. Alpha was set at 0.05 for all analyses.

Results

Effects of cytisine on ethanol intake

Regarding ethanol intake, the main effect of cytisine dose was significant on the 2nd day of treatment during both the 1st [$F(2,18) = 3.76$, $p = 0.043$] and 4th [$F(2,18) = 4.69$, $p = 0.023$] hrs post-injection. Dunnett t-test planned comparisons of the second day's data revealed that the highest dose of cytisine decreased ethanol intake during the first hr and first 4-hr post-injection periods (p 's < 0.044), whereas the effect of the lower dose of cytisine was significant only across the first 4-hr post-injection period ($p < 0.031$). As seen in Fig. 1, on the second day of cytisine treatment, both doses of cytisine decreased ethanol intake relative to saline during the first and/or first 4-hr post-injection period.

Effects of cytisine on percent ethanol preference

Regarding percent ethanol preference, the main effect of cytisine dose was significant for the 1-hr test period within each of the 3 test days [F -values > 4.65 , p -values < 0.024]. Dunnett's t-test planned comparisons for the 1-hr test period within each test day, revealed that the highest dose of cytisine significantly (p -values < 0.014) reduced percent ethanol preference, relative to control values, see Fig. 2. In addition, the lower dose of cytisine significantly reduced percent ethanol preference during the 1-hr test period on the second day of test ($p < 0.015$), see Fig. 2.

Effects of cytisine on water intake

Regarding water intake, the main effect of cytisine dose was significant each of the 3 test periods within each of the 3 test days, although some of these were marginally significant, $p = 0.05$, [F -values > 3.47 , p -values ≤ 0.05]. Dunnett t-test planned comparisons for each time point within each test day revealed that other than the first 4-hr post-injection period on the second test day ($p < 0.041$), only the highest dose of cytisine significantly increased water intake relative to control values (p -values < 0.036), and, as seen in Figure 3, this effect was consistent across test periods and test days. No significant differences were seen after the third cytisine test day. Cytisine had no significant effect on body weight (p -values > 0.50 , data not shown).

Effects of lobeline on ethanol intake

Regarding ethanol intake, the main effect of lobeline dose was significant each of the 3 test periods within each of the 3 test days [F -values > 3.88 , p -values < 0.041]. Dunnett t-test planned comparisons for each test period, within each test day, revealed that only the highest dose of lobeline significantly (p -values < 0.031) reduced ethanol intake, relative to control values, and this was observed for each test period within each test day, see Fig. 4. No significant differences were observed after the third day of lobeline testing.

Effects of lobeline on percent ethanol preference

Regarding percent ethanol preference, the main effect of lobeline dose was significant each of the 3 test periods within each of the 3 test days [F -values > 3.64 , p -values < 0.048], except for the 22-hr test period on the first day of test ($p > 0.40$). Dunnett's t-test planned comparisons

for each test period, within each test day, revealed that the highest dose of lobeline significantly (p-values < 0.038) reduced percent ethanol preference, relative to control values for each test period within each test day except for the 22-hr test period on the first day of test, see Fig. 5. In addition, the lower dose of lobeline significantly reduced percent ethanol preference during the 1- and 4-hr test periods on the last day of test (p-values < 0.044), see Fig. 5.

Effects of lobeline on water intake

Regarding water intake, the main effect of lobeline dose was significant for the 22-hr post-injection on the second day of test, each test period for the third day of test, as well as the 22-hr consumption on the second post-treatment day, see Fig. 6 [F-values > 3.89, p-values < 0.040]. Dunnett t-test planned comparisons for each test period within each test day revealed that, other than the 4-hr post-injection period on the second test day, only the highest dose of lobeline significantly (p-values < 0.035) increased water intake during each of these test periods, as well as test, or post-test, days relative to control values, see Fig. 6. On the third post-treatment day, water intake values for the 3 dose groups were indistinguishable from that observed during the baseline period (data not shown). Lobeline had no significant effect on body weight (p-values > 0.50, data not shown).

Discussion

The primary findings for the first part of this study were a significant main effect of cytosine treatment on the 2nd test day, with the 1.5 mg/kg dose significantly reducing ethanol intake at the 1 hr and 4 hr time-points, relative to saline, and the 0.5 mg/kg dose inducing a significant reduction at the 4 hr time-point (Fig. 1). On the other hand, lobeline treatment resulted in significant main effects of treatment for all 3 time points, within each test day, with the 5.0 mg/kg dose significantly reducing ethanol intake, relative to saline, at each time-point within each test day (Fig. 4). Thus, at higher doses both nAChR ligands independently suppressed ethanol intake, with a commensurate increase in water intake. Regarding ethanol preference, the highest doses of both cytosine and lobeline significantly reduced ethanol preference during the 1-hr test period of all three test days (Fig. 2 and Fig. 5). In addition, the highest dose of lobeline significantly reduced ethanol preference across the 4-hr test period of all three test days and across the 22-hr test period for the second and third test days (Fig. 5). Body weight was not affected by either ligand (data not shown). These findings provide further evidence that activity at nAChRs influences ethanol intake and these receptors are promising brain targets to treat alcohol abuse and dependence.

Cytosine is a partial agonist at alpha4beta2 and full agonist at beta4 nAChRs (Mineur et al., 2007) and selectively reduced ethanol consumption in HAD-2 rats while not reducing water consumption. Thus, cytosine did not have global adipsic effects, which, again, suggests that nAChRs play a critical role in regulating ethanol drinking. Previously, cytosine was reported to reduce binge ethanol drinking in mice (Hendrickson et al., 2009). Similarly, a derivative of cytosine (verenicline), a partial agonist for alpha4beta2 nAChRs has also been reported to reduce ethanol intake and ethanol-seeking behavior in Wistar and Long-Evans Rats (Steensland et al., 2007). A number of *in vitro* studies indicate that ethanol interacts directly with alpha4beta2 nAChRs where it acts as an allosteric modulator (Aistrup et al., 1999; Cardoso et al., 1999; Covernton & Connolly, 1997). In addition, several *in vivo* studies have demonstrated that alpha-4beta2 nAChRs are modulated by ethanol (Butt et al., 2004; Owens et al., 2003). However, most of the *in vitro* studies have shown that ethanol only modulates nAChR function at high (>100 mM) concentrations, with this alcohol concentration unlikely in the brain of the HAD-2 rats in the present study. Nevertheless, systemic ethanol increases DA release in the mesolimbic DA system, an effect that appears to require stimulation of nAChRs (Blomqvist et al., 1997; Tizabi et al., 2008; Erickson et al., 2009). Therefore, it is possible that cytosine

could modulate nAChRs in the mesolimbic DA system, which may mediate the reduction of ethanol consumption by attenuating the reinforcing effects of ethanol. We propose that cytosine reduces ethanol consumption in HAD-2 rats through its ability to act as a partial agonist at $\alpha 4\beta 2$ nAChRs in the VTA, which, in turn, reduces DA release in the Acb (Ericson et al., 2008). The recent report on verenicline, a cytosine derivative, ability to inhibit alcohol intake and alter ethanol-induced Acb-DA release (Steensland et al., 2007; Ericson et al., 2009), supports this hypothesis. However, future studies should delineate the exact neurochemical mechanisms underlying cytosine's effects on ethanol-associated behaviors in HAD-2 rats. Although cytosine has shown greater potency at $\alpha 4\beta 2$ nAChR subtypes compared with other combinations of subunits, cytosine's efficacy varies widely among different subunit combinations, such as $\alpha 3\beta 4$ nAChRs (Mihalak et al., 2006). Therefore, at this point we can not rule out the possibility that $\alpha 3\beta 4$ nAChRs also played a role in cytosine's effects on ethanol consumption (see Ericson et al., 2009).

Overall, the present findings using an animal model of alcoholism suggest that, despite its short duration of action, cytosine has clinical promise, similar to that of vernicline, in the management of ethanol consumption and ethanol-seeking behavior (Steensland et al., 2007). It may be efficacious in certain clinical populations when administered shortly before entering an environment where alcohol will be available. As indicated earlier, this nAChR ligand has been used to facilitate smoking cessation as well. There is a strong positive association between nicotine and ethanol addiction, and nAChRs represent a common point of action for ethanol and nicotine in their ability to activate the mesolimbic DA system, an important part of the brain reward system (Ericson et al., 2009). Thus, cytosine also holds promise as a medication for the treatment of ethanol and nicotine co-dependence.

In the present study, lobeline treatment resulted in a significant reduction of ethanol-drinking behavior (Fig. 4) and its preference over water (Fig. 5), compared to saline, in HAD-2 rats. Importantly, there did not appear to be a significant development of tolerance to these effects across the three days of treatment (Fig. 4 and Fig. 5). Similar attenuating effects of lobeline on ethanol consumption in mice have been reported recently (Farook et al., 2009). Lobeline has been considered to be an agonist, until recently, but with a unique pharmacological profile (Dwoskin and Crooks, 2002). Although classified as both an agonist and antagonist at nAChRs, lobeline has no structural similarity with nicotine and structure-function relationships do not suggest a common pharmacophore (Dwoskin and Crooks, 2002). Studies from the laboratory of Miller and colleagues (2001, 2004) indicate that lobeline inhibits nicotine-evoked Acb-DA release as well as Acb [3H]nicotine binding, thus acting as a potent antagonist at both $\alpha 3\beta 2$ and $\alpha 4\beta 2$ nAChRs.

Lobeline and its derivatives have also been found to diminish behavioral and neurochemical effects of psychostimulants (Benwell and Balfour, 1998; Harrod et al., 2001; Miller et al., 2001; Neugebauer et al., 2007). Taken together, these findings suggest that lobeline acts as an antagonist at nAChRs located in the mesolimbic DA system, which may mediate its ability to reduce ethanol drinking by HAD-2 rats. Several studies have indicated that mecamylamine, a nonselective nAChR antagonist, reduces ethanol drinking and low-dose ethanol-induced locomotor behavior in rodent models (Blomqvist et al., 1997; Ericson et al., 2008; Larsson et al., 2004), suggesting ligand blocking of nAChRs has the potential to reduce excessive ethanol intake. In general, these reports suggest that lobeline may attenuate the ethanol consumption behavior of HAD-2 rats via functional antagonism of $\alpha 4\beta 2$ and/or $\alpha 3\beta 2$ nAChR subtypes in the brain reward system.

In addition, lobeline has been found to bind to DA and vesicular monoamine transporters (cf. Miller et al., 2007; Teng et al., 1997; Wilhelm et al., 2004). Therefore, an interaction with these targets has been proposed to mediate lobeline's influence on neurochemical and behavioral

effects of psychostimulants (Dwoskin and Crooks, 2002). Recently, an *in vitro* study suggests that lobeline functions as a mu opioid receptor antagonist as well (Miller et al., 2007). Whether the effects of lobeline on ethanol-drinking behavior observed in this study are also mediated by these targets remains to be determined. Overall, the present results, combined with previous research, indicate that lobeline suppresses ethanol drinking behavior in HAD-2 rats via nAChRs expressed in brain reward neurocircuitry, where this ligand acts as a functional antagonist. However, the possibility of other molecular targets, as described above, can not be discounted. At present, lobeline has been proposed as a treatment for nicotine and methamphetamine addiction (Dwoskin and Crooks, 2002).

The question remains as to how cytosine, a partial agonist and lobeline, a putative antagonist would cause similar behavioral effects on ethanol consumption. With regard to cytosine, it has been shown that cytosine is a partial agonist at the $\alpha 4\beta 2$ nAChR and antagonizes the receptor's response to its endogenous neurotransmitter acetylcholine (Papke and Heinemann, 1994). Furthermore, cytosine has been found to inhibit DA, its metabolite and nicotine-induced increased mesolimbic DA turnover in the rat Acb (Coe et al., 2005). Taken together these results show effective reduction in nicotine-induced responses both *in vivo* and *in vitro* suggesting an antagonist profile at $\alpha 4\beta 2$ nAChRs. Therefore, cytosine could reduce ethanol intake by attenuating $\alpha 4\beta 2$ nAChR function, either by decreased efficacy of ligands at nAChRs or by inactivation of nAChRs via desensitization (Hogg and Bertrand, 2007; Picciotto et al., 2008). Similar cytosine-induced mechanism(s) may account for the reduction of ethanol consumption reported in the present study. Interestingly, cytosine and its derivative, varenicline were also found to be effective in reducing ethanol drinking (Steensland et al., 2007; Hendrickson et al., 2009). Moreover, cytosine's ability to block $\alpha 4\beta 2$ nAChR subtypes has been demonstrated elsewhere for other behavioral effects (Mineur et al., 2007). On the other hand, lobeline binds with nAChRs and acts as a nonselective competitive antagonist at $\alpha 4\beta 2$ and $\alpha 3\beta 2$ nAChRs (Teng et al., 1997; Dwoskin and Crooks, 2002). Lobeline has been found to inhibit nicotine-evoked $[3H]$ DA overflow from rat striatal slices (Miller et al., 2000). In addition, lobeline inhibits the effect of psychostimulants in behavioral and neurochemical assays. For example, lobeline reduces amphetamine-induced endogenous DA release from rat striatal slices (Miller et al., 2001), and it attenuates methamphetamine self-administration (Harrod et al., 2001). It is likely that lobeline inhibits these psychostimulant-induced effects through its activity at nAChRs, or possibly its ability to alter presynaptic dopamine storage and release (Miller et al., 2007). Although activity at opioid receptors (Miller et al., 2007) within the mesolimbic DA system can not be ruled out, the existing *in vitro* and *in vivo* findings suggest that lobeline displays the appropriate profile to act as a functional antagonist at nAChRs. The above suggests that both cytosine and lobeline act as functional antagonists at nAChRs, or via other substrates to interfere with ethanol self-administration. This may occur within the mesolimbic DA system. However, future studies will be required to delineate their mode of action.

The present results indicate that the inhibitory effect of lobeline on ethanol consumption is longer lasting compared to that of cytosine (see results). The differences in their duration of actions may be associated with their respective pharmacodynamic (see also above) and/or pharmacokinetic properties. For example, it has been reported that the plasma half-life for cytosine and lobeline are ~ 52 min and ~ 60 min, respectively, in various animal models (Miller et al., 2003; Tutka et al., 2006; Tzankova and Danchev, 2007). Therefore, it appears their rates of degradation are approximately the same. However, it has been reported that the hepatic metabolism of lobeline is slower than cytosine (Reavill et al., 1990; Tutka et al., 2006). Regarding blood-brain transport, Reavill et al. (1990) reported that the brain penetration index (brain/plasma concentration) for lobeline is approximately 10 fold higher than that for cytosine (3.2 vs. 0.28, respectively). This is consistent with lobeline's higher log *p* value, a measure of a compound's partition co-efficient, compared with that of cytosine (3.7 vs. 0.6, respectively;

Reavill et al., 1990). Thus, the efficacious pharmacodynamic and pharmacokinetic profile of lobeline may account for its longer lasting effects on ethanol intake. In contrast, moderately fast metabolism, poor absorption and limited brain penetration (c.f., Coe et al., 2005; Reavill et al., 1990) likely influence cytosine's transient effects on ethanol intake. Nevertheless further studies are necessary to elucidate the mechanisms of action for lobeline and cytosine's effects on ethanol drinking behavior, which may facilitate the development of a more efficacious pharmacological treatment for alcohol abuse and/or dependence.

It is noteworthy to mention that cytosine and its derivative varenicline were found to be effective in reducing ethanol consumption by mice and rats in drinking-in-the-dark (DID) and operant ethanol self-administration paradigms, respectively (Hendrickson et al., 2009; Steensland et al., 2007). Hendrickson et al (2009) investigated the effect of acute cytosine (1 or 3 mg/kg/day) using a DID procedure in mice. Although there was a dose-dependent effect, this was significant only with the 3 mg/kg dose. In the Steensland and colleagues' study (2007), the effects of both acute and chronic cytosine treatment (1 or 2 mg/kg/day) in continuous and intermittent operant access paradigms were investigated. Both treatments produced significant reductions in ethanol self-administration. Hendrickson and colleagues (2009) attributed the reduction of ethanol consumption to desensitization of relevant nAChR subtypes or alteration of DA signaling, due to modulation of mesolimbic DA release, before ethanol access. It is likely that the cytosine effects observed in the present study are due to similar mechanisms. In addition, cytosine and cytosine-based agents have been found to produce dose-dependent antidepressant effects in several animal models of depression (Mineur et al., 2007, 2009). While acute cytosine treatment produced significant effects in these behavioral models, chronic treatment with cytosine and/or its derivative was required for a robust effect (Mineur et al., 2007). Overall, it appears that there are some differences in the effect of acute cytosine, or its derivatives, treatment on ethanol drinking behavior (Hendrickson et al., 2009; Steensland et al., 2007) and within animal models of depression (Mineur et al., 2007, 2009). It is also possible that chronic treatment with cytosine and/or its derivatives is required for a robust effect as demonstrated in the latter studies. Alternatively, effective doses of the drug may depend on the behavioral tests used, such that dose-response curves are test specific. However, these issues remain to be confirmed in future studies.

In conclusion, the present findings support the development of lobeline, and cytosine, as potential medications for the treatment of alcohol abuse, dependence and possibly the treatment of co-dependence with nicotine and/or other drugs of abuse as well.

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Figure 1.

Effects of cytisine on ethanol intake in adult male high alcohol-drinking HAD-2 rats under continuous home-cage ethanol (15% and 30% concurrently) access conditions, with water and food freely available. There was a significant ($p < 0.05$) reduction in ethanol consumption during the 1-hr and 4-hr test periods of the second test day. *, indicates a significant ($p < 0.05$) difference from the saline control-values using a Dunnett's 2-tailed planned comparison t-test. It is noteworthy that the ethanol intakes observed in this study would be expected to result in peak blood alcohol concentrations exceeding 50 mg% (Bell et al., 2008; Murphy et al., 2002; Rodd et al., 2008).

Figure 2.

Effects of cytisine on percent ethanol preference in adult male high alcohol-drinking HAD-2 rats under continuous home-cage ethanol (15% and 30% concurrently) access conditions, with water and food freely available. There was a significant ($p < 0.05$) reduction in percent ethanol preference during the 1-hr test period on all three test days. *, indicates a significant ($p < 0.05$) difference from the saline control-values using a Dunnett's 2-tailed planned comparison t-test.

Figure 3.

Effects of cytisine on water intake in adult male high alcohol-drinking HAD-2 rats under continuous home-cage ethanol (15% and 30% concurrently) access conditions, with water and food freely available. There was a significant ($p < 0.05$) increase in water consumption induced by the highest dose on all three test days. At least for the second day of test, this may have been a compensation for the decreases in ethanol intake induced by cytisine. *, indicates a significant ($p < 0.05$) difference from the saline control-values using a Dunnett's 2-tailed planned comparison t-test.

Figure 4.

Effects of lobeline on ethanol intake in adult male high alcohol-drinking HAD-2 rats under continuous home-cage ethanol (15% and 30% concurrently) access conditions, with water and food freely available. There was a significant ($p < 0.05$) reduction in ethanol consumption induced by the highest dose of lobeline during the 1-hr, 4-hr and 22-hr test periods of all three test days. *, indicates a significant ($p < 0.05$) difference from the saline control-values using a Dunnett's 2-tailed planned comparison t-test. It is noteworthy that the ethanol intakes observed in this study would be expected to result in peak blood alcohol concentrations exceeding 50 mg% (Bell et al., 2008; Murphy et al., 2002; Rodd et al., 2008).

Figure 5.

Effects of lobeline on percent ethanol preference in adult male high alcohol-drinking HAD-2 rats under continuous home-cage ethanol (15% and 30% concurrently) access conditions, with water and food freely available. There was a significant ($p < 0.05$) reduction in percent ethanol preference, by the highest dose of lobeline, during the 1-hr and 4-hr test periods of all three test days as well as during the 22-hr test period on the second and third test days. *, indicates a significant ($p < 0.05$) difference from the saline control-values using a Dunnett's 2-tailed planned comparison t-test.

Figure 6.

Effects of lobeline on water intake in adult male high alcohol-drinking HAD-2 rats under continuous home-cage ethanol (15% and 30% concurrently) access conditions, with water and food freely available. There was a significant ($p < 0.05$) increase in water consumption induced by the highest dose on the second and third test days. For these test days, this may have been a compensation for the decreases in ethanol intake induced by lobeline. *, indicates a significant ($p < 0.05$) difference from the saline control-values using a Dunnett's 2-tailed planned comparison t-test.