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Discrimination of ethanol-nicotine drug mixtures in mice: dual interactive mechanisms of overshadowing and potentiation

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Abstract

Rationale—One possible basis for the proclivity of ethanol and nicotine co-abuse is an interaction between the discriminative stimulus (S^D) effects of each drug.

Objectives—The current work sought to assess the discriminative control of ethanol and nicotine cues in mice trained with drug mixtures and to determine whether interactive mechanisms of overshadowing and potentiation occur.

Methods—Male C57BL/6J mice were trained to discriminate ethanol (1.5 g/kg) alone or ethanol plus nicotine (0.4, 0.8 or 1.2 mg/kg base) in experiment 1, and nicotine (0.8 mg/kg) alone or nicotine plus ethanol (0.5, 1.0 or 2.0 g/kg) in experiment 2. Stimulus generalization of the training mixtures to ethanol, nicotine and the drug combination were assessed.

Results—Ethanol (1.5 g/kg) retained discriminative control despite the inclusion of a progressively larger nicotine dose within the training mixtures in experiment 1. Although the nicotine S^D was overshadowed by ethanol training doses > 0.5 g/kg in experiment 2, nicotine did potentiate the effects of low dose ethanol.

Conclusions—These findings are suggestive of dual mechanisms whereby ethanol (>0.5 g/kg) overshadows the S^D effects of nicotine, and at lower doses (< 1 g/kg) the salience of ethanol's S^D effects is potentiated by nicotine. These mechanisms may contribute to the escalation of concurrent drinking and smoking in a binge-like fashion.

Keywords

Drug discrimination; Discriminative stimulus; Ethanol; Alcohol; Nicotine; Overshadowing; Potentiation; Mice

Introduction

According to the Center for Disease Control and Prevention, smoking and heavy drinking are the leading and third leading causes of preventable death, respectively, in the United States. Although the probability of ethanol and nicotine dependence co-occurring in a sample population aged 15–54 by chance is 3.4%, the estimated incidence based on the National Comorbidity Survey is 6.9% (Anthony et al. 2000). Further, smoking rates among alcoholics (70–85%) have remained constant over the past 4 decades despite a steady decline in the general population (currently approximately 20%), suggesting that alcoholics

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are particularly vulnerable to nicotine co-abuse (Hays et al. 1999). This greater than expected degree of co-morbidity is suggestive of a synergistic or paradoxically antagonistic mechanism underlying the drug taking behavior of ethanol and nicotine. One possible basis for this phenomenon is an interaction between ethanol and nicotine at the level of their discriminative stimulus (S^D) effects.

Subjective effects of a drug that can act as a discriminative stimulus (S^D) reflect receptor-mediated activity that is commensurate with the pharmacological specificity of the training drug (Colpaert 1999). Ethanol produces a complex S^D with multiple elements contributing to the generation of its interoceptive cue (i.e., internal perception), including positive modulation of GABA_A and 5-HT_{1B/2C} receptor systems and antagonism of NMDA receptors (Grant 1994). In contrast, the discriminative cue for nicotine is primarily mediated by centrally-located nicotinic acetylcholine (nACh) receptors, as evidenced by studies demonstrating deficiencies in nicotine discrimination following either pharmacological antagonism (Gommans et al. 2000; Jutkiewicz et al. 2011) or genetic manipulation of nACh receptors (Shoaib et al. 2002). As pharmacologically distinct drugs, numerous studies have demonstrated that nicotine does not substitute for an ethanol cue (Bienkowski et al. 1998; Bienkowski and Kostowski 1998), and vice versa, ethanol does not generalize to a nicotine S^D (Korkosz et al. 2005; Le Foll and Goldberg 2005).

There is some evidence to indicate that either ethanol or nicotine pretreatment can modulate the discriminative control of the training (conditioned) drug. For instance, nicotine enhances ethanol discrimination by increasing levels of ethanol-appropriate responding in animals (Bienkowski and Kostowski 1998; Signs and Schechter 1986). Nicotine patch pretreatment was also found to enhance ethanol's subjective effects, such as feeling drunk and drug-induced euphoria (Kouri et al. 2004). The ability of ethanol to similarly modulate nicotine discrimination is less certain, with one study reporting that ethanol decreases the stimulus effects of nicotine (Korkosz et al. 2005). However, another study documented that in rats trained to discriminate 0.4 mg/kg nicotine from saline, ethanol (1.0 g/kg) increased the stimulus effects of 0.03 mg/kg nicotine and decreased the stimulus effects of 0.1 mg/kg nicotine without significantly shifting the nicotine dose-response curve (Le Foll and Goldberg 2005). In humans, ethanol augmented subjective measures of satisfaction and reward following cigarette smoking (Glautier et al. 1996; Rose et al. 2004). Therefore, although ethanol and nicotine do not generalize to one another, these drugs do appear to modulate one another's stimulus effects under certain conditions.

In previous drug discrimination work summarized above, examination of cross-generalization and modulatory interactions between ethanol and nicotine discrimination were undertaken when one drug was trained as the S^D and the other was given as a pretreatment to measure possible interactions. This experimental context likely deviates from the human condition because experience with (and conditioning to) ethanol and nicotine often times involve concurrent use over an extended period. To address this discrepancy, the current study investigated mixtures of ethanol and nicotine trained as a compound S^D . Studies with drug mixtures demonstrate that two pharmacologically-distinct drugs are largely discriminated based on each drug element as an independent stimulus (Stolerman et al. 1991). Further, the degree of stimulus control exhibited by each element over the mixture is dependent upon the training dose ratio of the two elemental drugs (Garcha and Stolerman 1989; Stolerman et al. 1987). In one classic example, midazolam was found to dose-dependently overshadow the discriminative control of nicotine as the magnitude of its ratio contribution to the mixture increased (Mariathasan and Stolerman 1993). Therefore, in the current work it was initially hypothesized that incremental increases in the training dose of nicotine (0.4 → 0.8 → 1.2 mg/kg) within the drug mixture would weaken (or overshadow) the acquisition and subsequent expression of the S^D effects of a

moderate ethanol training dose (1.5 g/kg). Unexpectedly, this dose of ethanol retained dominant stimulus control over the drug mixtures regardless of the co-conditioned nicotine training dose employed. These initial findings prompted a revision of our original supposition to consider the alternate hypothesis that ethanol dose-dependently overshadows nicotine S^D effects. Thus, a second experiment using a similar dose ratio strategy was carried out using a fixed nicotine training dose (0.8 mg/kg) in combination with escalating ethanol training doses (0.5 → 1.0 → 2.0 g/kg) to test this hypothesis.

Materials and Methods

Subjects

For the drug discrimination studies, male C57BL/6J (B6) mice weighing 19.8 ± 0.2 g and 23.9 ± 0.2 g upon arrival were used in experiment 1 ($n = 48$) and experiment 2 ($n = 40$), respectively. All mice were acquired from The Jackson Laboratory-West (Sacramento, CA) and were doubly housed in standard laboratory cages on a 12 h light/dark cycle (lights on at 0600 h). Mice were maintained at 90% of their free-feeding body weights by restricting daily access to food, which was given after each session. Water was freely available except during training and testing sessions. The local Institutional Animal Care and Use Committee approved all procedures in accordance with state and federal guidelines.

Apparatus and Discrimination Training

Standard 2-lever mouse conditioning chambers (MED Associates, St. Albans, VT) were used for training and subsequent testing, as described in detail elsewhere (Shelton and Grant 2002). Briefly, mice were initially trained to press one lever and then the opposite lever on a fixed ratio (FR)-1 response schedule to obtain 5 sec access to a dipper containing a sweetened milk solution (10% w/v sucrose, 10% w/v powdered milk) in daily 15-min sessions. Any responses on the inactive lever reset the FR requirement for the active lever. The response requirement was incrementally increased to FR-12. During the training sessions, one lever was designated as the drug-appropriate lever and the other lever as the non-drug (or saline) lever. Separate groups of mice were trained to discriminate drug or drug mixture from vehicle (saline; i.p.). In experiment 1, a fixed ethanol training dose alone or in combination with incrementally larger nicotine training doses was examined as follows: 1.5 g/kg ethanol (1.5E), 1.5 g/kg ethanol + 0.4 mg/kg nicotine (1.5E+0.4N), 1.5 g/kg ethanol + 0.8 mg/kg nicotine (1.5E+0.8N), and 1.5 g/kg ethanol + 1.2 mg/kg nicotine (1.5E+1.2N). In experiment 2, a fixed nicotine training dose alone or in combination with progressively larger ethanol training doses was tested as follows: 0.8 mg/kg nicotine (0.8N), 0.8 mg/kg nicotine + 0.5 g/kg ethanol (0.8N+0.5E), 0.8 mg/kg nicotine + 1.0 g/kg ethanol (0.8N+1.0E), and 0.8 mg/kg nicotine + 2.0 g/kg ethanol (0.8N+2.0E). For both experiments drug mixtures were administered as a single injection 10-min prior to session start, and mice were placed into inactive, dark chambers during pretreatment. Following 5 'forced-choice' sessions with saline and then the drug mixtures, drug and saline were administered on a double alternation schedule (saline, saline, drug, drug) with both levers available. Discrimination was acquired when mice responded 75% during the first FR and 80% during the entire session on the condition appropriate lever over 5 consecutive sessions. Additionally, mice were required to earn 15 dipper reinforcements per session.

Stimulus Generalization and Potentiation Test Procedures

Fifteen minute test sessions were conducted under non-extinction conditions. In both drug discrimination experiments, generalization curves were conducted for ethanol (0.32 – 3.0 g/kg) and then for nicotine (0.2 – 1.6 mg/kg). Test dose ranges for ethanol and nicotine were based on previous drug discrimination studies in mice (Shelton and Grant 2002; Shoaib et al. 2002). For all generalization tests, drugs were given 10-min prior to session start. A constant

dose of nicotine (0.8 mg/kg) was co-administered with various ethanol test doses for the potentiation tests conducted in experiment 2. In all cases, treatments were administered intraperitoneally (i.p.), and mice were required to demonstrate continued stimulus control (per above criteria) for a minimum of 3 consecutive sessions between adjacent tests. Latin-square designs were implemented to counter-balance the dose order of testing for each procedure.

Blood Ethanol Concentration (BEC)

A 20 μ l sample was collected from the medial saphenous vein 10-min post-injection of ethanol or ethanol-nicotine drug mixture. All samples were analyzed via ambient headspace sampling gas chromatography, as previously described (Finn et al. 2007).

Drugs

The stock solution for ethanol injections was prepared from 100% ethyl alcohol (Pharmco-Aaper, Shelbyville, KY) and diluted with saline to a final 20% v/v concentration. Nicotine hydrogen tartrate salt (Sigma-Aldrich, St. Louis, MO) was dissolved into either saline (for testing) or the stock ethanol solution (for training drug mixture) to the desired concentration. Nicotine doses are reported as base equivalent. All drugs were injected via an i.p. route.

Statistical Analysis

For the discrimination data, the percentage of drug-appropriate responding (% DAR) and response rate (total presses on both levers per 15-min session) were evaluated by repeated measure analysis of variance (ANOVA) with factors treatment (repeated) and training group. Additional dependent variables such as the number of session to meet discrimination criteria, discrimination accuracy, and ED₅₀ were also assessed with a 1-way ANOVA by training group. ED₅₀ values for ethanol and nicotine substitution were determined for each mouse using a sigmoidal curve-fit (4-parameter logistic equation) with variable slope. The occurrence of 80% DAR during testing was interpreted as full generalization to the training drug or drug mixture. Because no differences were detected between the dose-response curves constructed from post-drug versus post-saline test sessions (i.e., double determination of each test dose) for either % DAR or response rates, data were collapsed and the resultant curves for ethanol and nicotine are representative of the average of these two determinations. For the % DAR and response rates of each dose-response curve, the relevant baseline values during control training sessions with drug ('D') and non-drug (saline, 'S') were averaged and provided for statistical comparison to the test doses. If a mouse failed to obtain at least one dipper reinforcement during a test session then this data point was omitted from the analysis of % DAR, but was retained in the calculation of response rates. For the BEC evaluation in discrimination-trained mice a 1-way ANOVA on concentration (mg/ml) was run.

All statistical analyses were conducted with SigmaStat 3.5 software (Systat Software Inc., San Jose, CA). Tukey post-hoc tests were performed to assess pair-wise comparisons in the event that the relevant ANOVA determined a significant main effect or interaction between factors. In all cases, the threshold for statistical significance was set at $P < 0.05$. Prism version 5.0 (GraphPad Software, Inc., La Jolla, CA) was implemented to calculate ED₅₀s and construct all figures depicted.

Results

Drug Discrimination (Experiment 1): Fixed Ethanol Training Dose

Discrimination Acquisition and Accuracy—While some mice trained to discriminate 1.5E+0.8N and 1.5E+1.2N appeared to more rapidly achieve the discrimination criteria when compared to 1.5E trained mice (Fig 1A; see dashed line at 50%) there were no between-group differences in the mean number of sessions to acquisition (Fig 1B). The mean $\pm$ SEM number of sessions to meet discrimination criteria was 53.9 ± 3.5 , 53.5 ± 2.9 , 48.3 ± 1.8 , and 48.0 ± 3.0 for the 1.5E, 1.5E+0.4N, 1.5E+0.8N, and 1.5E+1.2N groups, respectively. Although there were no differences in discrimination accuracy between training groups, mice expressed slightly greater accuracy following drug ($97.64 \pm 0.26\%$) than following saline ($96.53 \pm 0.36\%$) control sessions [$F(1,42) = 6.97$; $P < 0.05$], based on all training sessions conducted throughout the testing phase.

Ethanol Dose-Response Curves—A significant main effect of dose [$F(7,294) = 450.04$; $P < 0.001$] on drug-appropriate responding (DAR) was detected for the ethanol dose-response curves. However, neither an effect of training group nor a dose $\times$ group interaction was detected. The 1.0 g/kg dose exhibited partial substitution (between 20 and 80% DAR) and generated significantly greater responding over saline control ('S') sessions ($P < 0.001$) whereas the 1.5, 2.0, and 3.0 g/kg doses fully substituted in all training groups and resulted in levels of DAR similar to those observed during drug control ('D') sessions (Fig 2A). Further, the ED_{50} values derived from the ethanol dose-response functions were not different between training groups: 0.86 ± 0.09 g/kg (1.5E), 0.80 ± 0.09 g/kg (1.5E+0.4N), 0.91 ± 0.06 g/kg (1.5E+0.8N) and 0.85 ± 0.13 g/kg (1.5E+1.2N). Therefore, presence of nicotine did not affect the ability of mice to discriminate the ethanol element (1.5 g/kg training dose) of the drug mixture cue.

A main effect of ethanol dose on response rates [$F(7,320) = 119.01$; $P < 0.001$] was determined during ethanol dose-response testing (Fig 2B). Overall, administration of training drug mixtures during control sessions ('D') tended to decrease response rates when compared to 'S' sessions ($P = 0.07$) throughout the testing phase. The 2 and 3 g/kg ethanol doses significantly reduced response rates ($P < 0.001$) by 23 and 83%, respectively, when compared to 'S' sessions. Neither an effect of training group nor a group $\times$ dose interaction for response rates met statistical significance, thereby indicating that the co-conditioning of nicotine within the training drug mixtures did not differentially alter the amount of total responses that resulted from ethanol substitutions.

Nicotine Dose-Response Curves—In contrast to ethanol substitutions, no test dose of nicotine generalized from the drug mixture in any of the four training groups (Fig 3A). A 2-way repeated measure (RM) ANOVA revealed a main effect of nicotine test dose [$F(7,282) = 204.21$; $P < 0.001$] as well as a training group $\times$ test dose interaction [$F(21,282) = 2.19$; $P < 0.01$] for DAR. All training groups demonstrated significantly greater DAR following 1.6 mg/kg nicotine versus their respective saline control sessions ($P < 0.05$ for 1.5E and 1.5E+0.4N groups; $P < 0.001$ for 1.5E+0.8N and 1.5E+1.2N groups). Further, mice trained to discriminate 1.5E+1.2N were the only subjects to show partial substitution with a nicotine test dose (i.e., 1.2 mg/kg) that matched their training condition (Fig 3A), as 0.4 and 0.8 mg/kg test doses produced less than 20% DAR in the 1.5E+0.4N and 1.5E+0.8N training groups, respectively. In the select few mice that nicotine did appear to fully generalize from the drug mixture (exhibited 80% DAR; 1–3 subjects/group) the corresponding ED_{50} values for substitution were greater than the dose of the nicotine element within the respective training drug mixtures (data not shown).

Nicotine substitutions exhibited a significant impact on response rates (Fig 3B), with a main effect of test dose [$F(7,287) = 57.43$; $P < 0.001$] and significant dose x training group interaction [$F(21,287) = 2.33$; $P < 0.001$] both realized. All training groups demonstrated significantly suppressed rates following administration of the 1.2 mg/kg ($P < 0.05$ for 1.5E + 0.8N and 1.5E + 1.2N groups; $P < 0.001$ for 1.5E and 1.5E + 0.4N groups) and 1.6 mg/kg (all $P < 0.001$) nicotine test doses when compared to their respective 'S' values. In general, the training groups conditioned with drug mixtures containing either no or low dose nicotine were the most susceptible to rate suppression during substitution testing. Specifically, the response rates of the 1.5E group with 1.2 and 1.6 mg/kg nicotine ($P < 0.05$) and the 1.5E + 0.4N group with 1.6 mg/kg nicotine ($P < 0.01$) were significantly lower than the respective rates reported for the 1.5E + 1.2N training group (Fig 3B). However, the 1.5E + 1.2N group also experienced an average response rate suppression of 32% following 1.6 mg/kg nicotine.

Nicotine Effects on BEC—Following evaluation of the nicotine dose-response curve, a BEC sample was collected from each mouse 10-min following the administration of its respective training drug mixture, a time point that matched the normal pretreatment latency that mice experienced between injection and start of a discrimination session. The mean $\pm$ SEM values were 1.46 ± 0.10 , 1.35 ± 0.10 , 1.17 ± 0.11 and 1.14 ± 0.12 mg/ml for the 1.5E, 1.5E + 0.4N, 1.5E + 0.8N and 1.5E + 1.2N groups, respectively. Although the addition of nicotine to the training drug mixtures was not found to significantly alter the plasma levels of ethanol by treatment group ($F(3,42) = 2.01$; data not shown), a linear regression analysis found a significant negative relationship between BEC and nicotine dose ($r = 0.35$; $P < 0.05$; $n = 46$).

Drug Discrimination (Experiment 2): Fixed Nicotine Training Dose

Discrimination Acquisition and Accuracy—There was a significant difference in the rate of acquisition between training groups [$F(3,31) = 3.21$; $P < 0.05$]. The 0.8N + 1.0E and 0.8N + 2.0E groups took significantly less sessions to meet acquisition criteria ($P < 0.05$) than the 0.8N group (Fig 4). The mean $\pm$ SEM number of sessions to criteria was 70.1 ± 7.1 , 49.6 ± 8.7 , 46.0 ± 3.0 and 51.0 ± 4.3 for the 0.8N, 0.8N + 0.5E, 0.8N + 1.0E and 0.8N + 2.0E groups, respectively. There was a significant main effect of group on discrimination accuracy during ongoing training [$F(3,31) = 7.81$; $P < 0.001$], as mice trained to discriminate 0.8N + 2.0E ($98.19 \pm 0.55\%$) were significantly more accurate during control sessions than 0.8N ($P < 0.001$; $94.77 \pm 0.61\%$), 0.8N + 0.5E ($P < 0.001$; $94.88 \pm 0.61\%$) and 0.8N + 1.0E ($P < 0.05$; $96.14 \pm 0.83\%$) trained mice. There was also a significant main effect of session type [$F(1,31) = 6.25$; $P < 0.05$], with mice expressing significantly greater accuracy during saline ('S'; $96.99 \pm 0.47\%$) than during drug ('D'; $95.27 \pm 0.54\%$) sessions ($P < 0.05$), based on training days conducted between test sessions.

Ethanol Dose-Response Curves—A 2-way RM ANOVA revealed main effects of both training group [$F(3,31) = 10.44$; $P < 0.001$] and ethanol test dose [$F(6,172) = 229.01$; $P < 0.001$] as well as a group x dose interaction [$F(18,172) = 6.48$; $P < 0.001$] on DAR. Overall, ethanol showed full generalization from the 0.8N + 1.0E and 0.8N + 2.0E training drug mixtures, partial generalization from 0.8N + 0.5E, and little to no generalization from 0.8N (Fig 5A). The 2.0 and 3.0 g/kg test doses fully substituted in the 0.8N + 1.0E and 0.8N + 2.0E groups, and resulted in significantly enhanced levels of DAR versus respective within-group 'S' values (all $P < 0.001$). Partial substitution by 2.0 g/kg was also observed in the 0.8N + 0.5E group, generating significantly great DAR when compared to the 'S' levels of this same group ($P < 0.001$). The 1.0 g/kg dose exhibited partial substitution in the 0.8N + 0.5E, 0.8N + 1.0E and 0.8N + 2.0E training groups, but significantly increased DAR over respective 'S' levels only in the two latter groups ($P < 0.001$). Differences between training groups were also noted during testing with 1.0 and 2.0 g/kg ethanol doses (Fig 5A). While all three drug mixture training groups had significantly greater DAR than the 0.8N training

group following administration of the 2.0 g/kg dose (all P s<0.01), the 0.8N+1.0E group also expressed significantly greater DAR than 0.8N-trained mice with the 1.0 g/kg test dose (P <0.01). ED₅₀ values were calculated for the 0.8N+1.0E and 0.8N+2.0E groups (0.75±0.09 and 1.11±0.11 g/kg, respectively), as ethanol fully generalized from these training drug mixtures. The mean ED₅₀ values of these two training groups were significantly different from one another [$F(1,13) = 5.79$; P <0.05].

A main effect of ethanol test dose [$F(7,217) = 134.72$; P <0.001] and a training group x dose interaction [$F(21,217) = 3.99$; P <0.001] were determined for response rates. Throughout the ethanol-dose response testing period, administration of the drug mixture during control sessions ('D') significantly decreased responses rates in the 0.8N+2.0E group (P <0.001) when compared to within-group 'S' sessions (Fig 5B). The 0.8N group was the most sensitive to the rate-suppressing effects of ethanol, as responding in this group was significantly reduced during testing with the 1.0 g/kg dose versus within-group 'S' levels (P <0.01). Further, the 0.8N+1.0E and 0.8N+2.0E training groups maintained higher response rates than the 0.8N group following administration of both 1.0 g/kg and 2.0 g/kg doses (all P s<0.05). While all training groups demonstrated significantly reduced response rates following exposure to the 2.0 g/kg (P s<0.05) and 3.0 g/kg (P s<0.001) doses when compared to respective 'S' values, the 0.8N+2.0E was clearly the least sensitive to the suppressive effects of the 2.0 g/kg test dose (Fig 5B).

Nicotine Dose-Response Curves—Main effects of training group [$F(3,25) = 18.67$; P <0.001], nicotine test dose [$F(7,172) = 117.78$; P <0.001], as well as a group x dose interaction [$F(21,172) = 7.18$; P <0.001] were detected for DAR. As expected, nicotine test doses of 0.8, 1.2 and 1.6 mg/kg fully substituted in the 0.8N group (Fig 6A), and all doses greater than 0.4 mg/kg produced significantly greater DAR when compared to 'S' levels for this group (all P s<0.001). The 0.8N+0.5E group exhibited a small rightward shift in its nicotine dose-response curve compared to the 0.8N group, with only the 1.2 and 1.6 mg/kg test doses producing full substitution and doses greater than 0.8 mg/kg showing DAR above within-group 'S' levels (all P s<0.001). Although nicotine resulted in only partial substitution in the 0.8N+1.0E and 0.8N+2.0E training groups, the 1.2 and 1.6 mg/kg test doses were able to generate DAR that significantly exceeded respective 'S' levels (all P s<0.01). Between group differences in DAR were also noted, with the 0.8N+1.0E and 0.8N+2.0E training groups exhibiting significantly reduced responding at all nicotine test doses of 0.4 mg/kg and greater (all P s<0.05) when compared to the 0.8N group (Fig 6A). Although ED₅₀ values for nicotine substitution tended to increase as the dose of ethanol in the training drug mixture increased (0.38±0.01, 0.52±0.18, 0.54±0.15 and 0.77±0.07 mg/kg nicotine in the 0.8N, 0.8N+0.5E, 0.8N+1.0E and 0.8N+2.0E groups, respectively), a significant difference between groups was not realized.

A 2-way RM ANOVA revealed a main effect of nicotine test dose [$F(7,175) = 23.27$; P <0.001] and a training group x dose interaction [$F(21,175) = 3.35$; P <0.001] for response rates. Consistent with the 'D' sessions during evaluation for the ethanol dose-response curves, administration of the drug mixture in the 0.8N+2.0E group also led to a significant suppression in response rates (P <0.001) versus within group 'S' levels during assessment of nicotine generalization (Fig 6B). The 0.8N+1.0E and 0.8N+2.0E training groups were most sensitive to the rate suppressing effects of nicotine, demonstrating significantly reduced response rates following administration of the 1.2 mg/kg test dose (P <0.05 and P <0.001, respectively) when compared to within-group 'S' levels. With the exception of the 0.8N+0.5E trained mice, all other training groups demonstrated significantly decreased rates following exposure to 1.6 mg/kg nicotine (all P s<0.01). Surprisingly, the 0.8N+0.5E was resistant to nicotine-induced suppression of response rates at all the doses tested (Fig 6B),

and showed significantly higher response rates versus the 0.8N group with the 1.6 mg/kg test dose ($P<0.05$).

Nicotine Potentiation of Ethanol-Appropriate Responding—With the exception of the 0.8N training group, co-administration of a constant nicotine dose (0.8 mg/kg; ‘N’) with various ethanol test doses resulted in elevated DAR when compared to the administration of ethanol doses alone (Fig 7, left panels). Main effects of test dose on DAR were determined for the 0.8N [$F(1,9) = 6.56$; $P<0.05$], 0.8N+0.5E [$F(5,10) = 15.00$; $P<0.001$], 0.8N+1.0E [$F(4,12) = 10.75$; $P<0.001$] and 0.8N+2.0E [$F(7,12) = 21.95$; $P<0.001$] training groups. Although administration of ethanol test doses in combination with ‘N’ in the 0.8N group generated similar DAR as that observed following ‘N’ alone (Fig 7A), a significant difference was noted for the 0.5 g/kg ethanol dose alone versus its combination with ‘N’ ($P<0.01$). In the 0.8N+0.5E training group (Fig 7C), 0.5 g/kg ethanol plus ‘N’ exhibited significantly greater DAR than ‘N’ alone ($P=0.01$). When administered in combination with ‘N’ the 0.32, 0.5 and 1.0 g/kg ethanol test doses were found to significantly escalate DAR over levels observed when the same test ethanol doses were evaluated alone (all $P<0.001$). In both the 0.8N+1.0E and 0.8N+2.0E training groups (Fig 7, panels E and G), co-administration of 0.5, 1.0 and 2.0 g/kg ethanol plus ‘N’ resulted in significantly increased DAR versus ‘N’ alone (all $P<0.05$). The 0.5 g/kg plus ‘N’ and 0.32 g/kg plus ‘N’ combinations in the 0.8N+1.0E group also resulted in significantly more DAR than the respective ethanol test doses alone ($P<0.05$). The comparison across the 0.5 g/kg ethanol dose level was unique for this training group (Fig 7E), as it was clear that the co-administration of ‘N’ resulted in a supra-additive effect on DAR (i.e., it was greater than the sum of the contributions of ‘N’ and 0.5 g/kg ethanol alone). The 0.5 g/kg plus ‘N’ combination similarly yielded a significantly elevated level of DAR when compared to the ethanol test dose alone in the 0.8N+2.0E group (Fig 7G).

The influence of ‘N’ on ethanol-appropriate responding was generally accompanied by additive effects of nicotine- and ethanol-induced rate suppression (Fig 7, right panels). Main effects of test dose on response rates were apparent for the 0.8N [$F(1,12) = 11.79$; $P<0.001$], 0.8N+0.5E [$F(5,12) = 18.98$; $P<0.001$], 0.8N+1.0E [$F(4,12) = 12.07$; $P<0.001$] and 0.8N+2.0E [$F(7,12) = 20.61$; $P<0.001$] training groups. Co-administration of the 2.0 g/kg ethanol test dose plus ‘N’ significantly reduced rates in all training groups when compared to treatment with ‘N’ alone (all $P<0.05$). In the 0.8N training group (Fig 7B) the combination of 0.5 g/kg ethanol plus ‘N’ produced significantly lower response rates than the ethanol test dose alone ($P<0.05$). A similar disparity was noted in the 0.8N+0.5E training group (Fig 7D) with 1.0 g/kg ethanol plus ‘N’ versus 1.0 g/kg ethanol alone ($P<0.001$). A treatment combination of ‘N’ plus ethanol test doses greater than 0.32 g/kg resulted in significantly lower rates in the 0.8N+1.0E and 0.8N+2.0E training groups (Fig 7, panels F and H) when compared to exposure to ethanol alone (all $P<0.05$).

Discussion

The current work identifies dual interactive mechanisms that appear to govern the discriminative stimulus (S^D) effects of ethanol-nicotine drug mixtures. Evidence indicated that ethanol training doses > 0.5 g/kg overshadow a nicotine S^D . First, progressively increasing the nicotine training dose magnitude in the drug mixture from 0 $\rightarrow$ 1.2 mg/kg was unable to surmount the overshadowing effect of a moderate ethanol training dose (1.5 g/kg; experiment 1). Second, progressively increasing the ethanol training dose magnitude in the drug mixtures from 0 $\rightarrow$ 2.0 g/kg resulted in a partial to full attenuation of a discriminable nicotine training dose (0.8 mg/kg; experiment 2). Collectively, these observations suggest that ethanol exerts dominant stimulus control over drug mixtures and that the nicotine cue is overshadowed by ethanol. A second interactive mechanism observed

was nicotine potentiation of ethanol S^D effects (see Fig 7). Ethanol dose-response curves exhibited significant leftward/upward shifts in the 0.8N+0.5E, 0.8N+1.0E and 0.8N+2.0E training groups when a constant dose of nicotine (0.8 mg/kg) was given in combination with ethanol test doses. Nicotine elicited a modest, non-significant reduction in BECs of discrimination-trained mice administered their respective drug mixture (experiment 1; data not shown), suggesting that the interactive mechanisms identified were unlikely influenced by an alteration in ethanol pharmacokinetics. In summary, developing a better understanding of overshadowing and potentiation mechanisms in the context of the S^D effects of ethanol-nicotine mixtures may help elucidate potential drug-drug interactions relevant to the greater than expected degree of ethanol and nicotine abuse co-morbidity.

One interactive mechanism identified was overshadowing, whereby a stimulus with normally sufficient salience is weakened by concurrent conditioning with one or more additional stimuli possessing even greater salience (Stolerman et al. 1991). Contrary to our original hypothesis, progressively increasing the magnitude of the nicotine training dose within drug mixtures containing 1.5 g/kg ethanol neither enhanced the discriminative control exhibited by nicotine nor diminished the salience of ethanol S^D effects (experiment 1). Based on previous studies in which the dose ratio of elements comprising the training drug mixture were parametrically examined, it was expected that the 0.8 mg/kg and 1.2 mg/kg training doses of nicotine would dose-dependently enhance the discriminability of nicotine in the mixture while reducing that of ethanol (Table 1, experiment 1). Two plausible explanations for the inability of nicotine to gain stimulus control within the drug mixtures evaluated in experiment 1 are as follows: 1) the magnitude of the nicotine training doses were insufficient, and 2) the 1.5 g/kg ethanol training dose was too large for nicotine to surmount. To address these concerns a follow-up study was conducted (experiment 2). This second drug discrimination study firmly established that 0.8 mg/kg nicotine alone (0.8N group) was capable of functioning as a S^D (see Figures 4A and 6A), a finding consistent with earlier reports that male B6 mice acquire nicotine discriminations with training doses of 0.8 and 1.2 mg/kg nicotine base (Gommans et al. 2000; Quarta et al. 2009; Shoaib et al. 2002; Stolerman et al. 1999). By extension, 0.8 and 1.2 mg/kg nicotine training doses in experiment 1 (1.5E+0.8N and 1.5E+1.2N training groups) should have been sufficiently large to gain at least partial stimulus control, except in the case that ethanol effectively overshadowed the nicotine cue. The incremental increase in ethanol training dose (0→2.0 g/kg) in combination with the fixed nicotine training dose (0.8 mg/kg) revealed that a threshold ethanol training dose between 0.5 and 1.0 g/kg results in a complete reversal of stimulus control over the drug mixtures, from a sufficient nicotine cue to a predominant ethanol one (Table 1, experiment 2). Interestingly, a high nicotine test dose (1.6 mg/kg) could achieve partial generalization from the drug mixtures in the 0.8N+1.0E (experiment 2) and 1.5E+0.8N (experiment 1) training groups, but not in the 0.8N+2.0E group (experiment 2), suggesting that overshadowing of the nicotine by ethanol was absolute with a training dose of 2.0 g/kg ethanol. Two observations regarding the estimated threshold ethanol dose required for overshadowing of the nicotine cue are worth noting. First, this threshold (between 0.5 – 1.0 g/kg ethanol) approximates the minimum training dose magnitude required to generate an ethanol S^D , as 1.0 g/kg is the lowest training dose successfully trained in mice to date (Becker et al. 2004). Second, the BECs observed in the groups receiving 1.5 g/kg ethanol training dose at the onset of a discrimination session (see results) hints that exposure to intoxication amounts of ethanol (>0.8 mg/ml) may be necessary for ethanol overshadowing of nicotine to occur.

Earlier work on an overshadowing mechanism in drug discrimination demonstrated complete reversal of stimulus control from nicotine to midazolam (Mariathasan and Stolerman 1993) and amphetamine to pentobarbital (Mariathasan et al. 1991) as the dose ratio was manipulated in favor of midazolam and pentobarbital, respectively. The

attenuation of the midazolam or pentobarbital cue by nicotine and amphetamine could not be readily explained by pharmacological antagonism, as the drug elements in each mixture were known to activate independent receptor systems. Rather, ‘overshadowing’ of one drug element by another appears to be a parsimonious explanation. In the current work, pharmacological antagonism of nicotine discrimination by ethanol is similarly unlikely, given that nicotine exerts its discriminative stimulus effects primarily through nicotinic acetylcholine (nACh) receptors and ethanol primarily through GABA_A, NMDA, and serotonergic receptor systems. Consistent with earlier studies (Bienkowski et al. 1998; Bienkowski and Kostowski 1998), it was observed that nicotine exhibited little to no generalization from ethanol (1.5E training group in Fig. 3A), and vice versa, ethanol showed no substitution for the nicotine S^D (0.8N group in Fig. 5A), thereby confirming that ethanol and nicotine are pharmacologically dissociable and likely perceived as distinct entities within the trained drug mixtures. The pharmacological profiles of the training drug mixtures support the conclusion that ethanol overshadowed the nicotine cue when the training dose of ethanol exceeded 0.5 g/kg, as the 0.8N+1.0E and 0.8N+2.0E training groups in experiment 2 and all three drug mixture groups in experiment 1 were found to predominantly possess ‘ethanol-like’ S^D properties.

The potentiating effect of nicotine on ethanol S^D effects in our drug mixture-trained mice (Fig 7) is consistent with previous reports investigating the influence of nicotine facilitation on substitution patterns of low ethanol test doses in rats trained to discriminate ethanol (Bienkowski and Kostowski, 1998; Signs and Schechter 1986). Specifically, in these prior studies nicotine doses of 0.3 – 0.4 mg/kg significantly enhanced ethanol-appropriate responding following administration of ethanol test doses ranging from 0.15 – 0.5 g/kg. Most notably, nicotine potentiated a 0.5 g/kg ethanol test dose in rats resulting in a shift from saline-like responding to full substitution for the training drug (Bienkowski and Kostowski, 1998). Potentiation of similar magnitude was observed with a 0.5 g/kg ethanol test dose in our mice trained to discriminate 0.8N+0.5E, 0.8N+1.0E and 0.8N+2.0E. The level of potentiation was clearly supra-additive in the 0.8N+1.0E training group (Fig 7), as nicotine administered prior to testing with 0.5 g/kg ethanol culminated in 90% DAR when compared to responding generated by 0.8 mg/kg nicotine (22%) and 0.5 g/kg ethanol (11%) alone. These similarities in nicotine potentiation of ethanol S^D effects across studies would suggest that co-conditioning of ethanol and nicotine during discrimination acquisition is not a pre-requisite for the demonstration of this interactive effect. At least one study in human subjects yielded congruent findings regarding this nicotine potentiating effect, as application of a transdermal nicotine patch enhanced several positive subjective effects of ethanol (Kouri et al. 2004). It is also noteworthy that two previous studies in rats have examined the related question of whether ethanol pretreatment influences the S^D effects of a trained nicotine cue, with one report indicating that ethanol (0.25 – 0.50 g/kg) partially antagonized nicotine (Korkosz et al. 2005) while a second report found little evidence that ethanol (0.1 – 1.0 g/kg) altered a nicotine S^D (Le Foll and Goldberg 2005). In contrast, available information from human subjects suggests the opposite; that ethanol pretreatment potentiates subjective/rewarding aspects of cigarette (nicotinized versus de-nicotinized) smoking such as smoking satisfaction, degree of stimulation, and relief of craving (Rose et al. 2004). Regardless of the uncertainty regarding the cross-generality of this potentiation mechanism, the current findings in conjunction with earlier work in rodents and humans demonstrate that nicotine potentiates the S^D effects of low dose ethanol.

In conclusion, it is important to note that ethanol itself is a stimulus complex generated by a composite of GABA_A, NMDA, and 5-HT, among other, receptor mechanisms (Grant 1994; Green and Grant 1998). In contrast, the nicotine cue appears to be primarily produced by activation of centrally-located nACh receptors (Wooters et al. 2009; Smith and Stoleran 2009). If ethanol is trained in combination with nicotine as a drug mixture, then ethanol

substitution for the drug mixture presumably activates many underlying mechanisms whereas a nicotine test dose may directly engage a single receptor system. This scenario differs from previous demonstrations of nicotine co-conditioning with a drug like midazolam, in which the discriminative control exhibited by each element is fully reversed by adjusting the dose ratio of the training mixture (Garcha and Stolerman 1989; Mariathasan and Stolerman 1993). It is noteworthy that nACh receptors are anatomically positioned to facilitate synaptic communication conducted by other neurotransmitters such as glutamate, GABA and 5-HT (Liechti and Markou 2008; Watkins et al. 2000). Further, emerging behavioral evidence suggests that nicotine recruits dopamine, 5-HT, glutamate, and cannabinoid systems in the generation of its S^D effects (for review, see Smith and Stolerman 2009). These insights raise the possibility that downstream effects of nACh receptor activation by nicotine may share common neurochemical pathways as those mediating the ethanol S^D complex. Viewed in this way, glutamate, GABA and serotonin neurotransmission may be the most salient aspects of ethanol/nicotine mixtures, and may overshadow the direct activity of nACh receptor in the discrimination. Future efforts will be required to discern the putative mechanistic roles of these neurotransmitter systems in animals co-conditioned with ethanol and nicotine. Nevertheless, the data in mice suggest that individuals that concurrently use ethanol and tobacco may experience a disproportionately greater conditioning to ethanol. The current findings also prompt speculation that co-abusers may resort to greater levels of smoking to compensate for an attenuated sensitivity to the S^D effects of nicotine in the presence of ethanol. This premise would be consistent with recent reports that smokers consume up to 3-fold more cigarettes than average during drinking episodes (Harrison et al. 2009; Witkiewitz et al. 2011).

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Fig. 1.

Acquisition of drug discrimination (experiment 1). Separate groups of mice ($n = 11-12$ /group) were trained to discriminate 1.5 g/kg ethanol (1.5E group; ○), 1.5 g/kg ethanol plus 0.4 mg/kg nicotine (1.5E+0.4N group; ■), 1.5 g/kg ethanol plus 0.8 mg/kg nicotine (1.5E+0.8N group; △), or 1.5 g/kg ethanol plus 1.2 mg/kg nicotine (1.5E+1.2N group; ▼) versus the non-drug condition (saline vehicle). In panel A, the cumulative percentage of mice meeting the criteria for discriminative control is depicted over successive training sessions. The intersection of each curve with the gray dashed line represents the number of training sessions at which 50% of mice acquired the discrimination. Each data point illustrated in panel B represents the number of sessions per subject to reach criteria whereas the solid gray

bars denote the mean of this measure per training group. No statistically significant differences between training groups were apparent.

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Fig. 2.

Stimulus generalization to ethanol (experiment 1). The percentage of drug-appropriate responding (% DAR, panel A) and corresponding response rates (panel B) following ethanol test doses in the 1.5E (○), 1.5E+0.4N (■), 1.5E+0.8N (△), and 1.5E+1.2N (▼) training groups are shown on a logarithmic scale. All data points shown are the mean $\pm$ SEM of $n = 11$ – 12 mice per training group. Measures recorded during all drug ('D') and non-drug (saline; 'S') training sessions that occurred between successive tests were averaged and used for statistical comparison to the test doses. Each ethanol test dose (including a saline test session; '0') was double-determined (once each following a drug and a vehicle training session) and the dose-response curves depicted represent the collapsed average of these two

determinations. *** $p < 0.001$ for all training groups versus respective within-group 'S' value.

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Fig. 3. Stimulus generalization to nicotine (experiment 1). The % DAR (panel A) and corresponding response rates (panel B) following nicotine test doses in the 1.5E (○), 1.5E + 0.4N (■), 1.5E + 0.8N (△), and 1.5E + 1.2N (▼) training groups are shown on a logarithmic scale. All data points shown are the mean $\pm$ SEM of $n = 11-12$ mice per training group. Each nicotine test dose was double-determined and the dose-response curves depicted represent the collapsed average of these two determinations. See Fig. 2 legend for additional details. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus respective within-group 'S' value; # $p < 0.05$ and ## $p < 0.01$ versus 1.5E+1.2N training group within respective test dose.

Fig. 4.

Acquisition of drug discrimination (experiment 2). Separate groups of mice ($n = 8-10$ /group) were trained to discriminate 0.8 mg/kg nicotine (0.8N group; ○), 0.8 mg/kg nicotine plus 0.5 g/kg ethanol (0.8N+0.5E group; ■), 0.8 mg/kg nicotine plus 1.0 g/kg ethanol (0.8N+1.0E group; △), or 0.8 mg/kg nicotine plus 2.0 g/kg ethanol (0.8N+2.0E group; ▼) versus the non-drug condition (saline vehicle). The cumulative percentage of mice meeting discrimination criteria over successive training sessions (panel A) and the number of sessions per subject to reach criteria (panel B) are depicted. See Fig. 1 legend for additional details. # $p < 0.05$ and ## $p < 0.01$ versus 0.8N training group mean.

Fig. 5. Stimulus generalization to ethanol (experiment 2). The % DAR (panel A) and corresponding response rates (panel B) following ethanol test doses in the 0.8N (○; n = 8), 0.8N+0.5E (■; n = 8), 0.8N+1.0E (△; n = 9) and 0.8N+2.0E (▼; n = 10) training groups are shown on a logarithmic scale. See Fig. 2 legend for additional details. *p < 0.05, **p < 0.01 and ***p < 0.001 versus respective within-group 'S' value; #p < 0.05, ##p < 0.01 and ###p < 0.001 versus 0.8N training group within respective test dose.

Fig. 6. Stimulus generalization to nicotine (experiment 2). The % DAR (panel A) and corresponding response rates (panel B) following nicotine test doses in the 0.8N (○; n = 4), 0.8N+0.5E (■; n = 7), 0.8N+1.0E (△; n = 8) and 0.8N+2.0E (▼; n = 10) training groups are shown on a logarithmic scale. See Fig. 2 legend for additional details. *p < 0.05, **p < 0.01 and ***p < 0.001 versus respective within-group 'S' value; #p < 0.05, ##p < 0.01 and ###p < 0.001 versus 0.8N training group within respective test dose.

Fig. 7.

Nicotine potentiation of ethanol S^D effects (experiment 2). The % DAR (left panels) and corresponding response rates (right panels) following ethanol test doses alone ($\diamond$) or in combination with 0.8 mg/kg nicotine ($\blacklozenge$) in the 0.8N (panels A-B; $n = 2$), 0.8N+0.5E (panels C-D; $n = 6$), 0.8N+1.0E (panels E-F; $n = 5$) and 0.8N+2.0E (panels G-H; $n = 8$) training groups are shown on a logarithmic scale. Combination treatment points were single-determined. 'N' represents values following treatment with 0.8 mg/kg nicotine alone. See Fig. 2 legend for additional details. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus within-group 'N' value; # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ versus respective ethanol test dose alone.

Table 1

Hypothesized overshadowing mechanism underlying ethanol-nicotine interactions. The discriminative control of nicotine (N) is overshadowed when concurrently conditioned with ethanol (E). The font size of each drug element reflects the relative discriminative control over the drug mixture that it possesses. The horizontal arrow denotes discrimination basis of the training drug or drug mixture.

Group	Training Drug(s)	Expected Outcome	Observed Outcome
<u>Experiment 1</u>			
1.5E	E	→ E	→ E
1.5E+0.4N	E + N	→ E _N	→ E
1.5E+0.8N	E + N	→ E N	→ E _N
1.5E+1.2N	E + N	→ E N	→ E _N
<u>Experiment 2</u>			
0.8N	N	→ N	→ N
0.8N+0.5E	N + E	→ N _E	→ N _E
0.8N+1.0E	N + E	→ N E	→ N _E
0.8N+2.0E	N + E	→ N E	→ N _E