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Contribution of NMDA glutamate and nicotinic acetylcholine receptor mechanisms in the discrimination of ethanol-nicotine mixtures

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Abstract

Ethanol and nicotine are commonly co-abused drugs, and the incidence of co-dependence is greater than would be expected based on the summed probability of dependence on each drug alone. Previous findings from our laboratory and others suggest that interactive mechanisms at the level of discriminative stimulus (S^D) effects may contribute to this co-abuse phenomenon. Specifically, ethanol overshadows the nicotine S^D whereas nicotine potentiates the stimulus salience of ethanol when the two drugs are conditioned as a drug mixture. The goal of the current study was to begin to delineate the pharmacological bases of these ethanol-nicotine interactions. Three groups of C57BL/6J mice were trained to discriminate 0.8 mg/kg nicotine + 0.5 g/kg ethanol (0.8N+0.5E), 0.8N+1.0E or 0.8N+2.0E. An NMDA receptor antagonist (MK-801) and three nACh receptor ligands were tested for their ability to generalize from or antagonize, respectively, the drug mixtures. MK-801 fully generalized from the 0.8N+1.0E and 0.8N+2.0E mixtures and partially generalized from 0.8N+0.5E. In contrast, nACh receptor ligands had minimal influence in blocking the perception of 0.8N+1.0E and 0.8N+2.0E mixtures, and only mecamylamine partially blocked 0.8N+0.5E. Reduced and enhanced contributions of nACh and NMDA receptors, respectively, in the discrimination of ethanol-nicotine mixtures may contribute to the overshadowing and potentiation phenomena previously observed.

Keywords

drug discrimination; drug mixture; ethanol; nicotine; NMDA receptor; nACh receptor; substitution; antagonism; overshadowing; mouse

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Introduction

Centrally-located $\alpha_4\beta_2^*$ nACh receptors produce the discriminative stimulus (S^D) effects of nicotine (Smith and Stolerman, 2009; Wooters et al., 2009) whereas NMDA, GABA_A and 5-HT_{1B/2C} receptor systems are crucial for the generation of an ethanol S^D (Grant, 1994). Numerous studies have also demonstrated that little to no cross-generalization occurs between ethanol and nicotine (Bienkowski et al., 1998; Bienkowski and Kostowski, 1998; Korkosz et al., 2005; Le Foll and Goldberg, 2005), suggesting that pharmacological distinct mechanisms underlie the S^D effects of each drug. Despite this, modulatory interactions between ethanol and nicotine occur. For instance, nicotine augments ethanol-appropriate responding in rats (Bienkowski and Kostowski, 1998) and the positive subjective effects of ethanol in men (Kouri et al., 2004) while the preponderance of evidence suggests that ethanol significantly attenuates a nicotine cue in rats (Kim and Brioni, 1995; Korkosz et al., 2005). A recent study that trained ethanol-nicotine drug mixtures in mice (Ford et al., 2012) may explain the greater than expected incidence of ethanol and nicotine co-dependence in the general population (see Anthony et al., 2000). Specifically, it was found that ethanol training doses > 0.5 g/kg overshadowed the S^D effects of a 0.8 mg/kg nicotine training dose, but the reciprocal ability of escalating nicotine training doses to exert stimulus dominance over ethanol was not realized. Overshadowing is a phenomenon whereby the salience of conditioned stimulus A is reduced due to the concurrent presence of stimulus B, and it has been demonstrated for both external sensory (Mackintosh, 1974) and interoceptive (Mariathasan and Stolerman, 1993) cues. The overshadowing that occurs during co-conditioning of ethanol and nicotine stimuli may represent a unique case, as ethanol itself is a complex stimulus with mixed, yet distinct, effects through multiple receptor systems (Grant and Colombo, 1993; also see above).

Previous work suggests that drug mixture discriminations enable the identification of pharmacological mechanisms pertinent to poly-drug abuse, particularly those underlying interactions (synergistic or antagonistic) between drug elements (Mariathasan et al., 1999; Stolerman et al., 1999a). Given that enhancement of glutamate release and NMDA receptor activation in the VTA are crucial for the ability of nicotine to elicit dopamine release within the nucleus accumbens (Mansvelder et al., 2002; Schilstrom et al., 2000), and that dopamine is an important mediator of the reinforcing and stimulant effects of nicotine (Mansvelder and McGehee, 2002) it seemed plausible that the NMDA antagonist-like properties of an ethanol S^D may be one mechanism by which ethanol overshadows the stimulus effects of nicotine. Thus, the goal of the current work was to further elucidate the pharmacological bases of ethanol-nicotine training drug mixtures in mice by evaluating 1) the ability of the NMDA receptor antagonist, dizocilpine (MK-801), to generalize from the drug mixtures, and 2) the influence of nACh receptor antagonists and a partial agonist to block discrimination of the compound drug cues. Based on our earlier work and that of others, it was hypothesized that if ethanol overshadows the nicotine cue then the drug mixtures would exhibit a predominantly 'ethanol-like' stimulus profile whereby MK-801 would fully substitute for the mixtures, but nACh receptor ligands would have little influence. Identification of receptor mechanisms that are emphasized and de-emphasized, respectively, in the perception

of combined ethanol-nicotine stimulant effects may help direct future treatment strategies for individuals with co-abuse problems.

Methods

Subjects

Male C57BL/6J mice were purchased from Jackson Laboratory-West (Sacramento, CA), housed two per cage upon arrival, and were acclimated to vivarium conditions for 2 weeks prior to experiment start. During all training and test phases mice were kept at 90% of their free-feeding body weight by restricting their daily food ration. The local Institutional Animal Care and Use Committee approved all procedures in accordance with state and federal guidelines.

Apparatus and drug discrimination procedure

Mice were trained to respond on a fixed ratio (FR) schedule of reinforcement for a sweetened milk solution (10% w/v sucrose + 10% w/v powdered milk) using standard 2-lever conditioning chambers (MED Associates, St. Albans, VT, USA), as previously described (Ford et al., 2012; Shelton and Grant, 2002). Once mice reliably responded under a FR-12 on each lever, one lever was designated as drug-appropriate (counterbalanced across chambers). Mice were then trained to discriminate one of three drug mixtures: 0.8 mg/kg nicotine + 0.5 g/kg ethanol (0.8N+0.5E group), 0.8 mg/kg nicotine + 1.0 g/kg ethanol (0.8N+1.0E group), and 0.8 mg/kg nicotine + 2.0 g/kg ethanol (0.8N+2.0E group). Nicotine doses were the base equivalent of nicotine tartrate. A double alternation treatment schedule was employed, and mice acquired discriminations based on the following criteria: 75% responding during the first FR and 80% responding during the entire session on the condition appropriate lever over 5 consecutive sessions. All sessions were 15-min and training drugs were administered (i.p.) 10-min prior to session start.

Mice were initially tested for the generalization of ethanol or nicotine alone from the drug mixtures (see summary in Figure 1 below; additional tests reported in Ford et al., 2012). Subsequently, a generalization curve was also established for the NMDA receptor antagonist, dizocilpine hydrogen maleate (MK-801), with test doses chosen based on previous drug discrimination studies in mice (Shoaib et al., 2002). Blocking tests were then performed, with nACh receptor ligands administered 10-min prior to the training drug mixture (i.e., 20-min prior to session start). The nACh receptor antagonists mecamylamine hydrochloride (MEC) and dihydro- β -erythroidine hydrobromide (DH β E), as well as the partial agonist varenicline tartrate (VAR), were assessed with dose selections based on previous reports in rodents (Ford et al., 2009; Gommans et al., 2000; Jutkiewicz et al., 2011; Stolerman et al., 1999b). All drugs were purchased from Sigma-Aldrich (St. Louis, MO, USA), and dissolved into saline. Test doses were administered according to a Latin-square design.

Data analysis

The dependent variables assessed were the percentage of drug-appropriate responding (% DAR) and response rate (total presses on both levers per 15-min session). Two-way

repeated-measures analysis of variance (RM ANOVA) with factors treatment (repeated) and training group was used for each variable. Full generalization from the training drug mixture occurred with 80% DAR. In the event that a subject was unable to obtain at least one reinforcer, the % DAR data point was omitted, but the concomitant response rate was included in the analyses. 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 when appropriate. Statistical significance was set at $P = 0.05$.

Results

Elemental contribution to drug mixtures

Historical control data for mice trained to discriminate 0.8 mg/kg nicotine alone (trained in tandem with current drug mixture training groups) clearly demonstrated that this training dose of nicotine served as a discriminative stimulus in our mouse model (Fig. 1). In the 0.8N +0.5E training group a 0.8 mg/kg test dose of nicotine exhibited near-full generalization from the mixture whereas the 0.5 g/kg test dose of ethanol was perceived as non-drug, suggesting that nicotine retained dominant stimulus over this drug mixture. As the dose ratio of the training mixture was adjusted in favor of ethanol, administration of 1 and 2 g/kg test doses of ethanol to the 0.8N+1E and 0.8N +2E training groups, respectively, revealed a shift towards ethanol as the dominant stimulus element in the mixtures. In these two latter groups, the same 0.8 mg/kg nicotine test dose showed little to no substitution for the training mixtures.

MK-801 generalization from drug mixtures

A significant training group $\times$ MK-801 dose interaction [$F(12,42) = 6.39$; $P < 0.001$] was found for %DAR. MK-801 produced full substitution in the 0.8N+1.0E and 0.8N+2.0E training groups, but not in subjects trained to discriminate 0.8N+0.5E (Fig 1A). Test doses of 0.1 mg/kg and greater in the 0.8N+1.0E group or 0.3 mg/kg and greater in the 0.8N+2.0E group generated levels of %DAR that significantly exceeded within-group 'S' session values (all $P_s < 0.05$). A significant group $\times$ MK-801 dose interaction [$F(12,46) = 2.47$; $P < 0.05$] was similarly detected for response rate. The 0.56 mg/kg dose suppressed responding in all three training groups versus respective 'S' session rates (all $P_s < 0.01$; Fig 1B). The 0.8N+1.0E training group was most sensitive to the rate suppressing effects of MK-801 (0.3 mg/kg; $P < 0.05$).

nACh receptor antagonism and drug mixture discrimination

A significant main effect of training group [$F(2,13) = 8.78$; $P < 0.01$] was found for %DAR during MEC treatment. Although no significant pair-wise comparisons within a given training group were found, %DAR in the 0.8N+0.5E group was significantly less than responding in the 0.8N+1.0E and 0.8N+2.0E training groups ($P < 0.01$ for each; Fig 2C). The large variance in %DAR expressed by the 0.8N+0.5E group was attributable to MEC completely blocking discrimination of the drug mixture in half the mice, but exerting no influence in the other half. significant main effects of training group [$F(2,13) = 4.71$; $P < 0.05$] and dose [$F(5,64) = 3.59$; $P < 0.01$], but no significant interaction, were determined for response rates during testing with MEC (Fig 2D). Overall, the 5.6 mg/kg MEC

attenuated response rates when compared to 'D' session values ($P<0.05$), and some 0.8N+0.5E trained mice were particularly sensitive.

Pretreatment with DH β E showed no evidence of blocking when compared to 'D' session %DAR levels (Fig. 2E), as evidenced by the absence of both a main effect of DH β E dose and a training group x dose interaction. Administration of DH β E prior to training drug mixtures yielded similar response rates as those observed following 'D' sessions (Fig. 2F). A trend towards a main effect of training group [$F(2,8) = 3.89$; $P=0.07$] was noted, likely reflecting the lower response rates observed in the 0.8N+2.0E training group.

VAR was without influence on %DAR in any training group when compared to 'D' session levels (Fig 2G). Significant main effects of VAR dose [$F(5,43) = 6.80$; $P<0.001$] and training group [$F(2,9) = 4.93$; $P<0.05$] were determined for response rates during VAR testing. Pretreatment with 5.6 mg/kg VAR significantly reduced response rates when compared to 'D' sessions ($P<0.001$; Fig 2H), and the 0.8N+2.0E training group expressed a significantly lower response rate when compared to the 0.8N+0.5E group ($P<0.05$).

Discussion

It was expected that the relative contribution of nACh and NMDA receptors to the discrimination of each ethanol-nicotine mixture would mirror the elemental contribution of the constituent drugs to the trained mixtures as assessed by substitution profiles of ethanol and nicotine test doses (as summarized in Fig. 1). In large part, this expectation was met with the 0.8N+1.0E and 0.8N+2.0E training groups. For these groups, ethanol was the dominant stimulus component that exhibited full generalization from the drug mixtures and the ethanol-like S^D effects of the NMDA receptor antagonist (MK-801) also fully generalized from the mixtures. Also consistent with this expectation, the nicotine cue was overshadowed by ethanol, as demonstrated by the absence of nicotine test dose generalization from the mixtures and nACh receptor antagonists and partial agonist were unable to block the perception of these drug mixtures. However, the crucial findings were that despite near-full nicotine test dose generalization from the 0.8N+0.5E mixture and the absence of any ethanol generalization from the same drug mixture (in Fig. 1) lower doses of the non-specific nACh receptor antagonist (MEC, Fig. 2C) were found to only partially block the drug mixture cue and MK-801 was able to partially substitute for 0.8N+0.5E (Fig. 2A).

Previous work has firmly established that MK-801 substitutes for ethanol in rats and mice (Colombo and Grant, 1992; Shelton and Grant, 2002). Further, MK-801 does not generalize from or antagonize the S^D effects of nicotine in rats (Kim and Brioni, 1995; Zakharova et al., 2005). These observations would suggest that this NMDA receptor antagonist produces 'ethanol-like' S^D effects, while being devoid of modulatory effects on a nicotine S^D . It is also known that the degree of MK-801 generalization from ethanol is related to training dose magnitude, with the progressively greater NMDA receptor antagonist-like representation being apparent as ethanol training dose increases from 1 to 2 g/kg in rat (Colombo and Grant, 1992). In the mouse, a 0.5 g/kg ethanol dose would likely be too small to generate a discriminative cue on its own, as 1 g/kg is the lowest training dose to successfully generate a

S^D in this species to date (Becker et al., 2004). Thus, 0.5 g/kg ethanol would not be expected to exhibit NMDA antagonist-like properties, and by extension, it was predicted that MK-801 would show no substitution for the 0.8N+0.5E mixture. However, a partial substitution (i.e., 40% DAR) was observed following treatment with 0.3 mg/kg MK-801 (Fig. 2A). One possible explanation for this finding is that an extensive history of concurrent conditioning with 0.8 mg/kg nicotine during discrimination training and maintenance enhanced the potency of the ethanol cue, at least in part by augmenting its NMDA receptor antagonist-like representation. This interpretation would be consistent with previous findings demonstrating that nicotine increased the stimulant effects of ethanol in rat, mouse and human subjects (Bienkowski and Kostowski, 1998; Ford et al., 2012; Kouri et al., 2004).

Pretreatment blockade with nACh receptor antagonists and a partial agonist were also tested to assess the 'nicotine-like' quality of the training drug mixtures. Previous reports in rodents indicated that the non-selective nACh receptor antagonist MEC dose-dependently blocks the S^D effects of nicotine (Jutkiewicz et al., 2011; Stoleran et al., 1999; Zakharova et al., 2005), but not those of ethanol (Bienkowski et al., 1998). Specifically, a 1.5 mg/kg MEC dose completely blocked a 0.8mg/kg nicotine training dose in C57BL/6 mice (Stoleran et al., 1999). Notably, this prior finding was observed with the same nicotine training dose and mouse strain as used in the current work. In the 0.8N+0.5E training group lower MEC doses (0.3 – 3.0 mg/kg) showed only a modest reduction in %DAR for this drug mixture, and this response was highly variable between subjects (Fig. 2C). Notably, MEC-induced attenuation of the 0.8N+0.5E cue was inversely dose-related as the highest MEC doses demonstrated no effect. One feasible explanation for this counter-intuitive observation is that μ M concentrations of MEC are known to block NMDA receptor channels, as evidenced by the ability of MEC to both antagonize NMDA receptor-mediated norepinephrine release in rat cortical slices (Gonzales et al., 1993) and protect against NMDA-induced excitotoxicity (Olney et al., 1987), presumably through a competitive binding at the phencyclidine site of the NMDA receptor. Thus, it is possible that high MEC test doses mimicked the ethanol-like S^D effects of NMDA receptor antagonism, resulting in a loss of its ability to block the 0.8N +0.5E drug mixture. In contrast, MEC had no effect in the 0.8N+1.0E and 0.8N+2.0E training groups, thereby confirming that nACh receptor activation served a negligible role in producing the S^D effects of these ethanol-nicotine drug mixtures. Further support for the conclusion that ethanol overshadowed the nicotine cue was indicated by the inability of the $\alpha_4\beta_2$ -selective antagonist DH β E or the partial agonist VAR to influence discrimination of the training drug mixtures examined (Fig 2E and 2G), despite ample evidence that these drugs either fully or partially attenuated the S^D effects of a nicotine training dose in rodents, respectively (Gommans et al., 2000; Jutkiewicz et al., 2011; LeSage et al., 2009). Although antagonism tests with DH β E upwards of 5.6 mg/kg exhibited no apparent effects on response rate (Fig. 2F), earlier work with the same mouse strain trained to discriminate an even larger nicotine training dose (i.e., 1.2 mg/kg) revealed that DH β E at 3 mg/kg fully blocked nicotine-appropriate responding (Gommans et al., 2000), suggesting that testing greater doses of this antagonist in the current work would not likely have selectively decreased %DAR for the ethanol-nicotine drug mixtures.

Earlier work with 'AND' discriminations (drug A + drug B versus saline) in rats demonstrated that ethanol is a compound stimulus with unique characteristics that differ

from other binary drug mixtures studied to date (Stolerman et al., 1999a; Harrison et al., 1998). Mainly, cross-generalization between ethanol and its component elements is partial or asymmetrical. However, ethanol will substitute for trained drug mixtures that include a combination of GABA_A receptor agonist + NMDA antagonist, with the greatest generalization found when the GABA_A receptor component is the predominant stimulus (Stolerman et al., 1999a). A separate study reported that pretreatment with diazepam, but not MK-801, dose-dependently attenuated the S^D effects of nicotine in nicotine-trained rats (Kim and Brioni, 1995). Taken together, these findings suggest that future efforts to additionally dissect the relative contribution of GABAergic and serotonergic receptor systems to the perception of ethanol-nicotine drug mixtures will lead to an even greater understanding of the mechanism by which ethanol overshadows nicotine.

In conclusion, following an extensive history of drug mixture administration ethanol overshadowed the S^D of nicotine, a phenomenon that is at least partially attributable to a diminished nACh receptor contribution and a stronger than expected NMDA receptor antagonist-like contribution. From a co-abuse perspective, these mechanisms may lead to greater nicotine self-administration to compensate for the reduction in the perceived stimulant effects of this drug. Further, the ability of nicotine to augment the S^D and reinforcing effects of ethanol (Ford et al., 2012; Le et al., 2003) could in turn result in greater ethanol consumption. These interpretations are consistent with reports that alcohol-dependent subjects exhibit both high probabilities of daily smoking and excessive amounts of drinking per episode (Anthony, 2000; Moss et al., 2007).

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Fig. 1.

Contribution of each drug element to the drug mixture discriminations. The % DAR, expressed as the mean + SEM of $n = 4-6$ mice per training group, is shown for each drug element that contributed to the drug mixture discrimination. The nicotine element (black bars) for each group reflects responding generated by a 0.8 mg/kg test dose whereas the ethanol element (white bars) corresponds to test dose magnitudes synonymous with the dose used in the training drug mixtures. The gray dashed line indicates the threshold for full generalization from the training drug or drug mixture.

Fig. 2. NMDA and nACh receptor involvement in the S^D effects of ethanol/nicotine drug mixtures. The % DAR and corresponding response rates generated by MK-801 test doses (panels A–B) or the training drug mixtures following 10-min pretreatment blockade with mecamylamine (MEC; panels C–D), dihydro- β -erythroidine (DH β E; panels E–F) or varenicline (VAR; panels G–H). All data points are the mean + SEM of $n = 3$ –6 mice/group and test doses are depicted on a logarithmic scale (y-axis). 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. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus within-group 'S' value.