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Nicotine acts in the anterior cingulate, but not dorsal or ventral hippocampus, to reverse ethanol-induced learning impairments in the plus-maze discriminative avoidance task

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Abstract

Introduction—The current study examines the role of the dorsal and ventral hippocampus, and anterior cingulate in the interactive effects of ethanol and nicotine on learning, anxiety, and locomotion in the plus-maze discriminative avoidance task, which allows dissociation of drug effects on each behavior.

Methods—At training, time spent in each of the arms of the elevated plus-maze was recorded for five minutes. Each time that the mouse entered the aversive enclosed arm, a light and white noise were turned on. At testing, no cues were turned on and time spent in each arm was recorded for three minutes. The effects of systemic ethanol (1.0 or 1.4 g/kg) and nicotine (0.35 µg/0.50 µl/side) infused into the anterior cingulate, dorsal and ventral hippocampus were examined, as were the interactive effects of systemic ethanol (1.0 g/kg) and nicotine (0.09 mg/kg) with the high-affinity nicotinic receptor antagonist dihydro-beta-erythroidine (DHβE) (18.0 µg/0.50 µl/side) infused into the anterior cingulate.

Results—Ethanol dose-dependently decreased anxiety, increased locomotion, and decreased learning. Anterior cingulate-infused nicotine decreased anxiety and reversed ethanol-associated learning deficits. Anterior cingulate-infused DHβE blocked reversal of ethanol-induced learning deficits by systemic nicotine. Dorsal hippocampus-infused nicotine reversed ethanol-induced anxiolysis and hyper-locomotion (1.4 g/kg) but produced no behavioral changes in ethanol-naïve mice. Ventral hippocampus-infused nicotine enhanced anxiolysis associated with 1.4 g/kg ethanol, but had no other effects.

Conclusions—The anterior cingulate is necessary and sufficient for nicotine reversal of ethanol-induced learning deficits. In addition, the anterior cingulate, dorsal hippocampus, and ventral hippocampus may mediate drug-induced changes in anxiety.

Keywords

nAChR; Learning; Anxiety; Motor Behavior; Alcohol

Introduction

Despite the well-known deleterious effects of both alcohol and nicotine on health, the co-abuse of these drugs remains a major societal issue. The National Institute of Health (2007) reports that nicotine-addicted smokers are four times more likely than non-smokers to also be addicted to alcohol and that alcoholics are three times more likely than the rest of the

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population to smoke cigarettes. This suggests that there may be common or interactive factors underlying abuse and co-abuse of these drugs. Specifically, alcohol and nicotine may interact to modulate affective processes, cognition, and/or activity levels. Ethanol decreases anxiety, while nicotine produces task-dependent changes in anxiety (Abreu-Villaca et al., 2008; Biala and Budzynska, 2006; Costall et al., 1989; Durcan and Lister, 1988) and previous work in our lab demonstrated that systemic ethanol and nicotine interact to alter anxiety (Gulick and Gould, 2009b). In addition, ethanol and nicotine have both independent and interactive effects on learning (Gould and Lommock, 2003; Gulick and Gould, 2008). Nicotine enhances learning but also reverses ethanol-induced learning deficits (Gould and Lommock, 2003; Gulick and Gould, 2008). Furthermore, ethanol and nicotine can alter locomotor activity; both drugs increase locomotion at low doses and decrease it at higher doses (Durcan and Lister, 1988; Elliott et al., 2004). The interactive effects of ethanol and nicotine on these behaviors could contribute to the co-use of these drugs (Ceballos, 2006); however, it is unclear whether the various effects of these drugs are due to actions on a single neural substrate, or whether different neural areas are involved in the effects of ethanol and nicotine on learning, anxiety, and locomotion.

Whereas numerous brain regions could be involved in the interactive effects of ethanol and nicotine on behavior, we were particularly interested in the dorsal hippocampus, ventral hippocampus, and anterior cingulate cortex. The dorsal hippocampus is involved in processing contextual information (Phillips and LeDoux, 1992) and direct infusion of nicotine into this area enhanced contextual fear conditioning (Davis et al., 2007). The ventral hippocampus may mediate anxiety (Engin and Treit, 2007) and the anterior cingulate is involved in multiple cognitive processes including aversive conditioning and behavioral sequencing in rodents (Chiba et al., 1997; Malin et al., 2007; Malin and McGaugh, 2006; Tang et al., 2005). Furthermore, previous work demonstrated that nicotine infusion into the anterior cingulate reversed ethanol-induced deficits in fear conditioning (Gulick and Gould, 2009a). However, in addition to a role in cognitive processes, the anterior cingulate is also involved in the perception of pain (Xu et al., 2008). Thus, it is possible that the interactive effects of nicotine and ethanol on fear conditioning are due to changes in pain perception; however, a previous study found no change in shock perception with alcohol or nicotine treatment (Gulick and Gould, 2009a). Two questions that remain unresolved are whether the involvement of the anterior cingulate in the interactive effects of ethanol and nicotine on learning extends to different types of learning and whether the anterior cingulate is involved in the effects of ethanol and nicotine on anxiety and locomotion. To answer the second question, it is necessary to use a task that allows assessment of anxiety, locomotion, and learning.

The plus-maze discriminative avoidance task (PMDAT) uses an elevated plus-maze consisting of two opposing, open arms and two opposing, enclosed arms to compare and contrast drug effects on anxiety, locomotion, and learning within subjects. During training, animals are free to explore all four arms but are conditioned to avoid one enclosed arm (the aversive arm) by the presentation of both light and white noise stimuli when they enter that arm. Time spent in the open arms, compared to time in all arms, is used as a measure of anxiety. Time in the aversive vs. non-aversive enclosed arms, both compared to time in all arms, is used as a measure of learning. The number of entries per minute into all arms is used as a measure of locomotion. On testing day, animals are again free to explore all four arms, with no presentation of the conditioning stimuli, and all three measures are again calculated. Previous work in our lab has demonstrated that ethanol and nicotine, given systemically, interact to alter behavior in the PMDAT, and that the differential effects of these drugs on anxiety, locomotion, and learning can be dissociated in this task (Gulick and Gould, 2009b).

In the current study, we compared the effects of nicotine infusion into the anterior cingulate cortex, dorsal hippocampus, and ventral hippocampus, alone and in combination with systemic ethanol administration, on learning, anxiety, and locomotion in the PMDAT. We also tested whether infusion of the high-affinity nicotinic acetylcholinergic receptor (nAChR) antagonist dihydro-beta-erythroidine (DH β E) into the anterior cingulate altered either the effects of ethanol alone or the interactive effects of ethanol and nicotine on the PMDAT.

Methods

Subjects

Male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were tested at 8–12 weeks of age (20–30 g). Mice were housed in groups of 4 per cage prior to surgery and individually housed after surgery. A 12-hr light–dark cycle (lights on at 7:00 am) was maintained, with all testing done between 9:00 am and 1:00 pm. Procedures were approved by the Temple University Institutional Animal Care and Use Committee.

Surgery

Mice were anesthetized using isoflurane gas (5% induction, 2% maintenance) and placed in a stereotaxic apparatus from David Kopf Instruments (Tujunga, CA). Bilateral stainless steel guide cannulae (C232G, 22 gauge; Plastics One, Roanoke, VA) were stereotaxically inserted and fixed to the skull with dental cement. Dummy cannulae (C232DC; Plastics One) were inserted into the guide cannulae to prevent clogging. Coordinates in reference to bregma were as follows: Dorsal Hippocampus: -1.7 mm posterior; ± 1.5 mm mediolateral; -2.3 mm ventral; Ventral Hippocampus: -2.8 mm posterior; ± 3.0 mm mediolateral; -4.0 mm ventral; Cingulate: $+2.0$ mm anterior; ± 1.5 mm mediolateral; -2.3 mm ventral (Paxinos and Franklin, 2001) (For placements, see Figure 1). To minimize post-operative pain, ketoprofen (2.0 mg/kg) was administered subcutaneously after surgery. Animals were allowed at least 5 days to recover before behavioral procedures began.

Drugs

Nicotine hydrogen tartrate salt and DH β E were procured from Sigma (St. Louis, MO). Ethanol was procured from Fisher Scientific (Pittsburgh, PA). Nicotine (0.09 mg/kg intraperitoneal (i.p.) injection or 0.35 μ g/0.50 μ l/side infusion, reported as freebase weight, based on Davis and colleagues (2007) and Davis and Gould (2006)) and DH β E (18.0 μ g/0.50 μ l/side infusion based on Davis and colleagues (2007)) were dissolved in physiological saline. Ethanol was diluted in saline and administered by i.p. injection only. Injection volume for systemic nicotine was 0.01 ml/g body weight, and for ethanol it was 20% vol/vol in saline. Controls received physiological saline. For direct infusions, mice were gently restrained and dummy cannulae were removed and replaced with 22 gauge infusion cannulae. Drugs were infused at a rate of 0.50 μ l per min and at an injection volume of 0.50 μ l per side. Infusion cannulae were attached to polyethylene tubing (PE50; Plastics One), which was attached to a 10 μ l Hamilton (Reno, NV) syringe. Drug administration was controlled by a micro-infusion pump (KDS 100; KD Scientific, New Hope, PA). Injection cannulae were left in place for 1 minute after infusion to allow drug diffusion away from the cannula tip. Nicotine was infused 5 minutes before training, and DH β E was infused 15 minutes before training.

Histology

Brains were rapidly dissected and placed in a 10% formalin solution (Fisher Scientific) for at least 24 hours before 60 μ m thick coronal sections were sliced at -18°C using a cryostat

and mounted on PLUS slides(Fisher Scientific). The sections were then stained with cresyl violet and cover-slipped, and placements were determined using a light microscope. Data from animals with incorrect cannula placements were excluded from statistical analysis (<5%).

Apparatus

The plus-maze discriminative avoidance task was performed using a modified elevated plus-maze that consisted of a wood base and grey Plexiglas floors and walls with no top. The entire maze was 36" off the ground, with two opposing enclosed (walled) arms ($12 \times 3 \times 6$ in, $L \times W \times H$, 5 lx) and two opposing open arms (12×3 in, $L \times W$, 10 lx). A 75-watt lamp (600 lx) was placed directly over one enclosed arm, and a speaker connected to a noise generator (85 dB) was placed directly below the same arm.

Procedure

For all experiments, groups consisted of 6–9 animals per condition. For the training session, each mouse was placed in the center of the apparatus and, for five minutes, the time spent in each arm was recorded by the researcher. The number of arm entries per minute was also recorded. Each time that the mouse entered the aversive arm, the 75-watt light and the 85 dB white noise were turned on by the researcher. Both cues were turned off when the mouse exited the aversive arm. The testing session was conducted 24 hours later. For the testing session, each mouse was returned to the center of the apparatus and, for three minutes, the time spent in each arm was recorded, as was the number of arm entries per minute. No cues were turned on during the testing session. The maze was cleaned with 70% ethanol before each training and testing session. This procedure, including the length of the testing session, was based on Silva and Frussa-Filho (2000). The length of the training session is shorter than that employed by Silva and Frussa-Filho to avoid a ceiling effect on learning that was apparent with a longer training session.

We first investigated how the infusion of nicotine into the anterior cingulate, alone or concomitant with systemic ethanol administration, altered behavior in the PMDAT at both training and testing; ethanol (1.0 or 1.4 g/kg, i.p.) was injected 15 minutes before training, and nicotine (0.35 μ g/0.50 μ l/side) was infused 5 minutes before training. In Experiment 2, to determine whether high-affinity nAChRs in the anterior cingulate are necessary for nicotine to reverse ethanol-induced impairments, we infused DH β E into the anterior cingulate concomitant with systemic ethanol and nicotine administration; DH β E (18.0 μ g/0.50 μ l/side) was infused 15 minutes before training, ethanol (1.0 g/kg, i.p.) was injected 15 minutes before training, and nicotine (0.09 mg/kg, i.p.) was injected 5 minutes before training. Whereas both the 1.0 and 1.4 g/kg doses of ethanol disrupted leaning, the 1.0 g/kg dose was chosen for experiments examining the effects on DH β E on ethanol-induced learning deficits because, unlike the 1.4 g/kg, the 1.0 g/kg dose was not anxiolytic. In Experiment 3, we tested if DH β E alone would alter the effects of systemic ethanol administration on learning in the PMDAT; DH β E (18.0 μ g/0.50 μ l/side) was infused 15 minutes before training and ethanol (1.0 g/kg, i.p.) was injected 15 minutes before training. In Experiments 4 and 5, we investigated how the infusion of nicotine into the dorsal or ventral hippocampus, alone or concomitant with systemic ethanol administration, altered behavior in the PMDAT; ethanol (1.0 or 1.4 g/kg, i.p.) was injected 15 minutes before training, and nicotine (0.35 μ g/0.50 μ l/side) was infused 5 minutes before training. No drugs were administered at testing. Injection and infusion times were based on previous research (Davis et al., 2007; Gulick and Gould, 2007).

Scoring

Scoring was done by a researcher blinded to the drug treatment. Entry into an arm was counted when all four paws crossed into that arm. Time in the center area was not counted toward any measure. Total number of entries per minute into all arms was used as an index of locomotion. Percent time in the aversive enclosed arm vs. percent time in the non-aversive enclosed arm (time in arm/time in all arms) was used as an index of learning (increased time in the aversive enclosed arm and decreased time in the non-aversive enclosed arm = decreased learning), and percent time in the open arms (time in open arms/time in all arms) was used as an index of anxiety (increased time in the open arms = decreased anxiety).

Statistical Analyses

Differences between groups were analyzed by one-way or two-way (drug treatment (group) and arm as independent variables for learning; drug treatment as independent variable(s) for anxiety; drug treatment (group) and day as independent variables for locomotion) analysis of variance (ANOVA). Levene's test for homogeneity of variance was used to determine appropriate post-hoc tests, and either Tukey's (homogeneous variability) or Duncan's (non-homogeneous variability) post-hoc tests were run at the level of $p < 0.05$. Statistics were calculated with SPSS (Version 14; SPSS, Chicago, IL).

Results

Experiment 1: Anterior Cingulate-Infused Nicotine and Systemic Ethanol

Percent time in the aversive arm—In our first set of experiments, we examined the interaction of ethanol and anterior cingulate-infused nicotine on learning in the PMDAT. At training, there was a significant effect of group on percent time in the closed arms, $F(5,69) = 11.90$, $p < 0.001$, a significant effect of arm, $F(1,73) = 329.16$, $p < 0.001$, and a significant group by arm interaction, $F(5,69) = 10.09$, $p < 0.001$. Tukey's post-hoc tests revealed that all groups spent significantly less time in the non-aversive arm than saline controls ($p < 0.05$) except the group administered 1.0 g/kg ethanol. In addition, all groups spent more time in the non-aversive arm than in the aversive arm ($p < 0.05$) (Figure 2A). At testing, there was no effect of group, but there was a significant effect of arm, $F(1,73) = 349.38$, $p < 0.001$, as well as a group by arm interaction, $F(5,69) = 20.15$, $p < 0.01$, on percent time in the closed arms. Tukey's post-hoc tests revealed that both groups administered ethanol alone spent more time in the aversive arm than saline controls ($p < 0.05$), while the ethanol alone groups and the 1.4 g/kg ethanol plus nicotine group all spent less time in the non-aversive arm than saline controls ($p < 0.05$). In addition, all groups except the group administered 1.4 g/kg ethanol alone spent significantly more time in the non-aversive arm than in the aversive arm ($p < 0.05$) (Figure 2B). Thus, ethanol administered at training impaired learning at testing, and nicotine infused into the anterior cingulate at training reversed this learning deficit.

Percent time in the open arms—We next examined whether systemic ethanol and nicotine infusion into the anterior cingulate interact to alter anxiety. During training, there was a significant effect of ethanol on percent time in the open arms, $F(2,34) = 34.11$, $p < 0.001$, and a significant effect of nicotine on percent time in the open arms, $F(1,35) = 16.78$, $p < 0.001$, but no ethanol by nicotine interaction. Tukey's post-hoc tests revealed that the groups administered 1.4 g/kg ethanol alone, nicotine alone, and 1.4 g/kg ethanol with nicotine all spent significantly more time in the open arms compared to saline controls ($p < 0.05$) but there were no other group differences (Figure 2C). There was also a significant effect of ethanol on percent time in the open arms during testing, $F(2,34) = 11.87$, $p < 0.001$, but no effect of nicotine and no ethanol by nicotine interaction. Tukey's post-hoc tests revealed that both groups administered 1.4 g/kg ethanol at training spent more time in the

open arms compared to saline controls at testing, regardless of nicotine treatment ($p < 0.05$), but there were no other group differences (Figure 2D). Thus, both anterior cingulate nicotine and 1.4 g/kg ethanol decreased anxiety at training, and 1.4 g/kg ethanol administered at training decreased anxiety at testing, but there were no additive effects of the treatments.

Total entries into all arms—Finally, we examined changes in locomotor activity. In order to determine whether overall locomotor activity changed between training and testing as well as between groups, we used a two-way ANOVA with group and day (training or testing) as independent variables. There was a significant effect of group on entries per minute, $F(5,68) = 7.17$, $p < 0.001$, and an effect of day, $F(1,72) = 5.15$, $p < 0.05$, but no group by day interaction. Tukey's post-hoc tests revealed that only the group administered 1.4 g/kg ethanol alone had significantly more entries than saline controls at training ($p < 0.05$). Furthermore, the group administered 1.4 g/kg ethanol alone at training had significantly more entries than saline controls during testing ($p < 0.05$); all other groups were similar to controls (Figure 2E–F). Thus, the higher dose of ethanol increased locomotion at training and testing, and nicotine infused into the anterior cingulate at training blocked this motor enhancement at both training and testing. Although ethanol and cingulate-infused nicotine interact to alter both learning and locomotion, the findings that both 1.0 g/kg and 1.4 g/kg ethanol impair learning but nicotine only interacts with the higher dose of ethanol to modulate motor behavior suggest that changes in motor behavior cannot account for the interaction of ethanol and nicotine on learning.

Experiment 2: Anterior Cingulate-Infused DH β E with Systemic Nicotine and Systemic Ethanol

Percent time in the aversive arm—Nicotine infused into the anterior cingulate reversed ethanol-induced learning deficits, suggesting that the anterior cingulate is sufficient for nicotine to reverse these deficits. To determine if the anterior cingulate is also necessary for nicotine to reverse ethanol-induced learning deficits, we blocked high-affinity nAChRs via infusion of the nAChR antagonist DH β E into the anterior cingulate and administered ethanol and nicotine systemically. At training, there was a significant effect of arm, $F(1,53) = 81.81$, $p < 0.001$ on percent time in the closed arms, but no effect of group and no group by arm interaction. Tukey's post-hoc tests revealed that all groups spent more time in the non-aversive arm than in the aversive arm ($p < 0.05$). Furthermore, the group administered ethanol plus nicotine and the group administered ethanol plus nicotine and DH β E both spent less time in the non-aversive arm than saline controls ($p < 0.05$) (Figure 3A). At testing, there was no effect of group, but a significant effect of arm, $F(1,53) = 14.08$, $p < 0.001$ on percent time in the closed arms, and a significant group by arm interaction, $F(3,51) = 22.53$, $p < 0.001$. Tukey's post-hoc tests revealed that the groups administered saline or ethanol plus nicotine spent significantly more time in the non-aversive arm than in the aversive arm ($p < 0.05$), and the ethanol alone group and the group administered ethanol plus nicotine and DH β E spent significantly less time in the non-aversive arm and more time in the aversive arm than saline controls ($p < 0.05$) (Figure 3B). Thus, DH β E infused into the anterior cingulate at training blocked the reversal of ethanol-induced learning deficits by nicotine.

Percent time in the open arms—We next examined whether DH β E infusion into the anterior cingulate interacts with systemic ethanol and nicotine to alter anxiety. During training, there was a significant effect of group on percent time in the open arms, $F(3,23) = 4.74$, $p < 0.01$. Tukey's post-hoc tests revealed that the group administered ethanol with nicotine and DH β E spent a significantly higher percentage of time in the open arms compared to saline controls ($p < 0.05$) but there were no other group differences (Figure 3C). There was no effect of group on percent time in the open arms during testing (Figure 3D).

Thus, there was an interaction of anterior cingulate-infused DH β E with systemic nicotine and ethanol such that administration of all three drugs reduced anxiety at training.

Total entries into all arms—Finally, we examined changes in locomotor activity. There was no effect of day on mean entries per minute, no effect of group, and no group by day interaction (Figure 3E–F). Thus, DH β E did not interact with ethanol and nicotine to alter locomotion at training or testing.

Experiment 3: Anterior Cingulate-Infused DH β E with Systemic Ethanol

Percent time in the aversive arm—Because DH β E infusion into the anterior cingulate modulated the interactive effects of ethanol and nicotine in the PMDAT, we also examined whether DH β E infusion into the anterior cingulate could interact with ethanol to alter behavior in the absence of nicotine. At training, there was a significant effect of arm on percent time in the closed arms, $F(1,50) = 138.18$, $p < 0.001$, but no effect of group and no group by arm interaction. Post-hoc tests revealed that all groups spent significantly more time in the non-aversive arm than in the aversive arm at training ($p < 0.05$), but there were no significant differences between groups (Figure 4A). At testing, there was no effect of group, but there was a significant effect of arm, $F(1,50) = 213.15$, $p < 0.01$, and group by arm interaction, $F(3,48) = 14.88$, $p < 0.01$, on percent time in the closed arms. Post-hoc tests revealed that the group administered ethanol alone spent more time in the aversive arm and less time in the non-aversive arm than saline controls ($p < 0.05$), but there were no other significant differences from controls (Figure 4B). In addition, all groups except the group administered ethanol alone spent more time in the non-aversive arm than in the aversive arm ($p < 0.05$). Thus, ethanol impaired learning expressed at testing, and DH β E infused into the anterior cingulate at training reversed this learning deficit.

Percent time in the open arms—We next examined whether systemic ethanol and DH β E infusion into the anterior cingulate also interact to alter anxiety. During training, there was a significant effect of DH β E on percent time in the open arms, $F(1,25) = 5.01$, $p < 0.05$ but no significant effect of ethanol and no ethanol by DH β E interaction. However, Tukey's post-hoc tests revealed no significant differences from controls; the effect of DH β E was due to both DH β E-treated groups spending a non-significantly greater amount of time in the open arms (Figure 4C). There was no significant effect of ethanol or DH β E on percent time in the open arms during testing and no ethanol by DH β E interaction (Figure 4D). Thus, there were no interactive effects of ethanol and anterior cingulate-infused DH β E.

Total entries into all arms—Finally, we examined changes in locomotor activity. There was a significant effect of group, $F(3,48) = 2.99$, $p < 0.05$, on mean entries per minute, and a significant effect of day, $F(1,50) = 6.17$, $p < 0.05$, but no group by day interaction. Post-hoc tests revealed no differences between groups (Figure 4E–F). Thus, neither ethanol nor DH β E produced significant changes in locomotion at training or testing.

Experiment 4: Dorsal Hippocampus-Infused Nicotine and Systemic Ethanol

Percent time in the aversive arm vs. non-aversive arm—We first examined the interaction of ethanol and dorsal hippocampus-infused nicotine on learning in the PMDAT. At training, two-way ANOVA revealed an effect of group, $F(5,75) = 3.03$, $p < 0.05$, and arm, $F(1,79) = 102.8$, $p < 0.001$, on percent time in the closed arms, as well as a significant group by arm interaction, $F(5,75) = 4.49$, $p < 0.05$. Tukey's post-hoc tests revealed that only the group administered 1.4 g/kg ethanol spent significantly less time in the non-aversive arm than saline controls ($p < 0.05$). In addition, all groups except the group administered 1.4 g/kg ethanol spent significantly more time in the non-aversive arm than in the aversive arm ($p < 0.05$) (Figure 5A). Thus, the higher dose of ethanol disrupted arm preference at training.

At testing, two-way ANOVA revealed an effect of group, $F(5,75) = 4.65$, $p < 0.05$, and arm, $F(1,79) = 70.40$, $p < 0.001$, on percent time in the closed arms, as well as a significant group by arm interaction, $F(5,75) = 36.36$, $p < 0.001$. Tukey's post-hoc tests revealed that all ethanol-treated groups spent more time in the aversive arm and less time in the non-aversive arm than saline controls, regardless of nicotine treatment ($p < 0.05$), while the group administered nicotine alone was not significantly different from controls. In addition, only the groups administered saline or nicotine alone spent significantly more time in the non-aversive arm than in the aversive arm ($p < 0.05$) (Figure 5B). Both doses of ethanol administered at training impaired learning at testing, and this impairment was not reversed by nicotine infusion into the dorsal hippocampus.

Percent time in the open arms—In addition to the interaction of systemic ethanol and dorsal hippocampus-infused nicotine on learning, we also examined drug effects on anxiety. During training, there was a significant effect of ethanol on percent time in the open arms, $F(2,37) = 18.44$, $p < 0.001$, and a significant effect of nicotine on percent time in the open arms, $F(1,38) = 14.34$, $p < 0.001$, but no ethanol by nicotine interaction. Tukey's post-hoc tests revealed that the group administered 1.4 g/kg ethanol alone spent significantly more time in the open arms compared to saline controls ($p < 0.05$) but there were no other group differences (Figure 5C). There was also a significant effect of ethanol on percent time in the open arms during testing, $F(2,37) = 4.10$, $p < 0.05$, as well as a significant effect of nicotine, $F(1,38) = 6.32$, $p < 0.05$, and a significant ethanol by nicotine interaction, $F(2,37) = 3.63$, $p < 0.05$. Tukey's post-hoc tests revealed that the group administered 1.4 g/kg ethanol alone spent more time in the open arms compared to saline controls at testing ($p < 0.05$), but there were no other group differences (Figure 5D). The higher dose of ethanol administered at training decreased anxiety at training and testing, and dorsal hippocampus nicotine blocked this effect on both days.

Total entries into all arms—Finally, we examined changes in locomotor activity. There was a significant effect of group, $F(5,74) = 6.51$, $p < 0.01$, and day, $F(1,78) = 16.09$, $p < 0.001$, on entries per minute, but no group by day interaction. Tukey's post-hoc tests indicated that only the group administered 1.4 g/kg ethanol alone had significantly more entries than saline controls at training ($p < 0.05$). Furthermore, the group administered 1.0 g/kg ethanol alone and both groups administered 1.4 g/kg ethanol at training had significantly more entries than saline controls during testing regardless of nicotine infusion ($p < 0.05$); no other groups were significantly different from controls (Figure 5E–F). Thus, the higher dose of ethanol administered at training increased locomotion at training and testing, and nicotine infused into the dorsal hippocampus at training reversed this motor enhancement at training but not at testing.

Experiment 5: Ventral Hippocampus-Infused Nicotine and Systemic Ethanol

Percent time in the aversive arm vs. non-aversive arm—Finally, we examined the interaction of ethanol and ventral hippocampus-infused nicotine on learning in the PMDAT. At training, there was a significant effect of group, $F(5,79) = 4.83$, $p < 0.001$, and of arm, $F(1,83) = 26.72$, $p < 0.001$, on percent time in the closed arms, and a significant group by arm interaction $F(5,79) = 3.06$, $p < 0.05$. Tukey's post-hoc tests revealed no significant differences from the saline control group. However, only the groups administered saline, 1.0 g/kg ethanol alone, or nicotine alone spent significantly more time in the non-aversive arm than in the aversive arm ($p < 0.05$) (Figure 6A). At testing, there was no effect of group, but a significant effect of arm, $F(5,79) = 40.69$, $p < 0.001$, and a group by arm interaction, $F(5,79) = 11.58$, $p < 0.01$, on percent time in the closed arms. Tukey's post-hoc tests revealed that all ethanol-treated groups spent more time in the aversive arm and less time in the non-aversive arm than saline controls regardless of nicotine treatment ($p < 0.05$), while the group

administered nicotine alone was not significantly different from controls. Furthermore, only the saline and nicotine-alone groups spent significantly more time in the non-aversive arm than in the aversive arm ($p < 0.05$) (Figure 6B). As with nicotine infusion into the dorsal hippocampus, the ethanol-induced learning deficit was not reversed by nicotine infusion into the ventral hippocampus.

Percent time in the open arms—We also examined the interaction of ethanol and ventral hippocampus-infused nicotine on anxiety. During training, there was a significant effect of ethanol on percent time in the open arms, $F(2,35) = 26.37$, $p < 0.001$, and a significant effect of nicotine on percent time in the open arms, $F(1,36) = 14.50$, $p < 0.001$, as well as an ethanol by nicotine interaction, $F(2,35) = 6.08$, $p < 0.01$. Tukey's post-hoc tests revealed that the groups administered 1.4 g/kg ethanol, 1.4 g/kg ethanol with nicotine, and 1.0 g/kg ethanol with nicotine all spent a significantly higher percentage of time in the open arms compared to saline controls ($p < 0.05$); in addition, the group administered 1.0 g/kg ethanol with nicotine spent significantly more time in the open arms than the group administered 1.0 g/kg ethanol alone ($p < 0.05$) (Figure 6C). There was no effect of ethanol or nicotine, and no significant ethanol by nicotine interaction on time in the open arms at testing (Figure 6D). The higher dose of ethanol decreased anxiety at training, and the lower dose of ethanol plus nicotine infusion into the ventral hippocampus produced a synergistic decrease in anxiety at training.

Total entries into all arms—Finally, we examined changes in locomotor activity. There was no effect of group or day on mean entries per minute, and no significant group by day interaction (Figure 6E–F). Thus, neither ethanol nor ventral hippocampus-infused nicotine altered locomotion in this experiment.

It should be noted that the percent time spent in the aversive and non-aversive arms does vary between experiments. These experiments took place over an extended period of time, and it is likely that environmental factors may have caused these changes in behavior. Nonetheless, the pattern of drug effects remains constant across studies, suggesting that the environmental factors did not alter the outcome of any experiments.

Discussion

In the current study, we demonstrate that the interactive effects of ethanol and nicotine on behavior depend upon diverse neural substrates. Specifically, we demonstrate that the anterior cingulate, but not the dorsal or ventral hippocampus, is necessary for nicotine to reverse ethanol-induced learning deficits. Furthermore, antagonism of high-affinity nAChRs – receptors with a high-affinity for nicotine, such as the $\alpha 4\beta 2$ nAChR that is found in high density throughout brain areas such as the anterior cingulate and hippocampus – in the anterior cingulate blocks the reversal of ethanol-induced learning deficits by nicotine. Together, these results suggest that high-affinity nAChRs in the anterior cingulate are necessary and sufficient for nicotine to reverse ethanol-induced learning deficits.

The present results extend previous work in our lab demonstrating that nAChRs in the anterior cingulate are necessary and sufficient for reversal of ethanol-induced deficits in fear conditioning by nicotine (Gulick and Gould, 2009a) by demonstrating that high-affinity nAChRs in the anterior cingulate are involved in the interactive effects of ethanol and nicotine on different types of learning. Fear conditioning is an aversive Pavlovian classical conditioning task that uses a foot-shock as an unconditioned stimulus, whereas the PMDAT is an operant form of learning that does not involve nociceptive stimuli. Because one function of the anterior cingulate may be the perception of pain (Xu et al., 2008), the involvement of the anterior cingulate in the nicotine amelioration of ethanol-induced deficits

in fear conditioning could be attributed to changes in foot-shock perception. However, this argument cannot explain the results from the current study. Thus, the current findings and our prior work (Gulick and Gould, 2009a) indicate that the anterior cingulate may modulate the interactive effects of ethanol and nicotine on cognitive processes. This is in line with work by other researchers demonstrating that the anterior cingulate is involved in attention, cognitive control (Cohen et al., 1999; Posner and Rothbart, 1998; Ridderinkhof et al., 2004), and learning (Bissiere et al., 2008; Han et al., 2003).

Identifying the anterior cingulate as a site of action for the effects of nicotine on ethanol-induced deficits in learning is only the first step in identifying the underlying neural substrates. Nicotine both activates and desensitizes nAChRs (for review, Picciotto et al., 2008), and the current results suggest that desensitization of nAChRs in the anterior cingulate may play an important role in the amelioration of ethanol-induced deficits in learning. In the absence of nicotine, infusion of the high-affinity nAChR antagonist DH β E into the anterior cingulate produces a reversal of ethanol-induced learning deficits comparable to that produced by nicotine. This supports the hypothesis that both nicotine and DH β E reverse ethanol-induced learning deficits by inactivating nAChRs (Gulick and Gould, 2009a). Interestingly, ethanol potentiates nAChR activity (Marszalec et al., 1999). Thus, ethanol may impair learning by over-activating nAChRs in the anterior cingulate, and a decrease in nAChR activity via nicotine desensitization of the receptors or via inhibition of the receptors by DH β E could return the system to a level of activity optimal for learning. However, this needs to be determined.

Whenever there are drug effects on a learning and memory task, it is always possible that the effects are due to changes in non-learning related processes such as anxiety or locomotion; multiple lines of evidence from the present study suggest this is not the case. Nicotine and ethanol altered anxiety but these effects varied with the brain area examined. Nicotine infusion into the anterior cingulate decreased anxiety when administered alone but did not interact with ethanol to modulate anxiety. Nicotine infusion into the dorsal hippocampus blocked the anxiolytic effect of ethanol, while nicotine infusion into the ventral hippocampus had a synergistic anxiolytic effect, decreasing anxiety when co-administered with a non-anxiolytic dose of ethanol. A number of studies have demonstrated that nicotine can both increase (Biala and Budzynska, 2006) and decrease (Costall et al., 1989) anxiety; one explanation for the diverse effects of nicotine on anxiety is that the processes altered by nicotine may depend both upon the task examined and the level of anxiety. For example, Seth and colleagues (2002) demonstrated that nicotine infused into the dorsal hippocampus has no effect during high-anxiety and low-anxiety tasks, but is anxiogenic during moderate-anxiety tasks, while nicotine infused into the dorsal raphe nucleus is anxiolytic regardless of the anxiety level in the task. The current findings support the possibility that nicotine infusion into the anterior cingulate, dorsal hippocampus, and ventral hippocampus mediate distinct effects on anxiety. However, the absence of an interactive effect of ethanol and nicotine infused into the anterior cingulate on anxiety suggests that changes in anxiety do not underlie the reversal of ethanol-induced learning deficits by nicotine seen in the current study.

Finally, nicotine infusion into the anterior cingulate and dorsal hippocampus, but not the ventral hippocampus, blocked the ethanol-induced increase in locomotion. Two findings from the current research support the position that the interactive drug effects that modulate locomotion do not underlie changes in learning. The dorsal hippocampus is not involved in the reversal of ethanol-induced learning deficits by nicotine, yet is involved in the effects of nicotine on ethanol-induced increases in locomotion. Furthermore, 1.0 and 1.4 g/kg ethanol impaired learning but only 1.4 g/kg ethanol enhanced locomotion at training, suggesting that different processes underlie the drug-induced changes in learning and in locomotion.

In conclusion, the anterior cingulate cortex is necessary and sufficient for nicotine to reverse ethanol-induced learning deficits, and these interactive effects of ethanol and nicotine are independent of drug-induced changes in anxiety or locomotion. The effects of ethanol and nicotine on learning may involve changes in activation of high-affinity nAChRs in the cingulate cortex. Furthermore, the anterior cingulate, as well as the dorsal hippocampus and ventral hippocampus, may be involved in the effects of nicotine and ethanol on anxiety.

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References

- Abreu-Villaca Y, Nunes F, do EQ-GF, Manhaes AC, Filgueiras CC. Combined exposure to nicotine and ethanol in adolescent mice differentially affects anxiety levels during exposure, short-term, and long-term withdrawal. *Neuropsychopharmacology*. 2008; 33:599–610. [PubMed: 17460612]
- Biala G, Budzinska B. Effects of acute and chronic nicotine on elevated plus maze in mice: involvement of calcium channels. *Life sciences*. 2006; 79:81–88. [PubMed: 16458932]
- Bissiere S, Plachta N, Hoyer D, McAllister KH, Olpe HR, Grace AA, Cryan JF. The rostral anterior cingulate cortex modulates the efficiency of amygdala-dependent fear learning. *Biological psychiatry*. 2008; 63:821–831. [PubMed: 18155183]
- Ceballos NA. Tobacco use, alcohol dependence, and cognitive performance. *The Journal of general psychology*. 2006; 133:375–388. [PubMed: 17128957]
- Chiba AA, Kesner RP, Gibson CJ. Memory for temporal order of new and familiar spatial location sequences: role of the medial prefrontal cortex. *Learning & memory (Cold Spring Harbor, NY)*. 1997; 4:311–317.
- Cohen RA, Kaplan RF, Moser DJ, Jenkins MA, Wilkinson H. Impairments of attention after cingulotomy. *Neurology*. 1999; 53:819–824. [PubMed: 10489048]
- Costall B, Kelly ME, Naylor RJ, Onaivi ES. The actions of nicotine and cocaine in a mouse model of anxiety. *Pharmacology, biochemistry, and behavior*. 1989; 33:197–203.
- Davis JA, Kenney JW, Gould TJ. Hippocampal alpha4beta2 nicotinic acetylcholine receptor involvement in the enhancing effect of acute nicotine on contextual fear conditioning. *J Neurosci*. 2007; 27:10870–10877. [PubMed: 17913920]
- Durcan MJ, Lister RG. Time course of ethanol's effects on locomotor activity, exploration and anxiety in mice. *Psychopharmacology*. 1988; 96:67–72. [PubMed: 2906444]
- Elliott BM, Faraday MM, Phillips JM, Grunberg NE. Effects of nicotine on elevated plus maze and locomotor activity in male and female adolescent and adult rats. *Pharmacology, biochemistry, and behavior*. 2004; 77:21–28.
- Engin E, Treit D. The role of hippocampus in anxiety: intracerebral infusion studies. *Behavioural pharmacology*. 2007; 18:365–374. [PubMed: 17762507]
- Gould TJ, Lommock JA. Nicotine enhances contextual fear conditioning and ameliorates ethanol-induced deficits in contextual fear conditioning. *Behav Neurosci*. 2003; 117:1276–1282. [PubMed: 14674846]
- Gulick D, Gould TJ. Interactive effects of ethanol and nicotine on learning in C57BL/6J mice depend on both dose and duration of treatment. *Psychopharmacology*. 2007
- Gulick D, Gould TJ. Interactive effects of ethanol and nicotine on learning in C57BL/6J mice depend on both dose and duration of treatment. *Psychopharmacology*. 2008; 196:483–495. [PubMed: 17968532]
- Gulick D, Gould TJ. The hippocampus and cingulate cortex differentially mediate the effects of nicotine on learning versus on ethanol-induced learning deficits through different effects at nicotinic receptors. *Neuropsychopharmacology*. 2009a; 34:2167–2179. [PubMed: 19404242]

- Gulick D, Gould TJ. Interactive effects of ethanol and nicotine on learning, anxiety, and locomotion in C57BL/6 mice in the plus-maze discriminative avoidance task. *Neuropharmacology*. 2009b; 57:302–310. [PubMed: 19500603]
- Han CJ, O'Tuathaigh CM, van Trigt L, Quinn JJ, Fanselow MS, Mongeau R, Koch C, Anderson DJ. Trace but not delay fear conditioning requires attention and the anterior cingulate cortex. *Proceedings of the National Academy of Sciences of the United States of America*. 2003; 100:13087–13092. [PubMed: 14555761]
- Malin EL, Ibrahim DY, Tu JW, McGaugh JL. Involvement of the rostral anterior cingulate cortex in consolidation of inhibitory avoidance memory: interaction with the basolateral amygdala. *Neurobiology of learning and memory*. 2007; 87:295–302. [PubMed: 17079169]
- Malin EL, McGaugh JL. Differential involvement of the hippocampus, anterior cingulate cortex, and basolateral amygdala in memory for context and footshock. *Proceedings of the National Academy of Sciences of the United States of America*. 2006; 103:1959–1963. [PubMed: 16446423]
- Marszalec W, Aistrup GL, Narahashi T. Ethanol-nicotine interactions at alpha-bungarotoxin-insensitive nicotinic acetylcholine receptors in rat cortical neurons. *Alcoholism, clinical and experimental research*. 1999; 23:439–445.
- Paxinos, G.; Franklin, KBJ. *The mouse brain in stereotaxic coordinates*. 2. Academic Press; San Diego: 2001.
- Phillips RG, LeDoux JE. Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. *Behav Neurosci*. 1992; 106:274–285. [PubMed: 1590953]
- Picciotto MR, Addy NA, Mineur YS, Brunzell DH. It is not “either/or”: activation and desensitization of nicotinic acetylcholine receptors both contribute to behaviors related to nicotine addiction and mood. *Progress in neurobiology*. 2008; 84:329–342. [PubMed: 18242816]
- Posner MI, Rothbart MK. Attention, self-regulation and consciousness. *Philosophical transactions of the Royal Society of London*. 1998; 353:1915–1927. [PubMed: 9854264]
- Ridderinkhof KR, Ullsperger M, Crone EA, Nieuwenhuis S. The role of the medial frontal cortex in cognitive control. *Science (New York, NY)*. 2004; 306:443–447.
- Tang J, Ko S, Ding HK, Qiu CS, Calejesan AA, Zhuo M. Pavlovian fear memory induced by activation in the anterior cingulate cortex. *Molecular pain*. 2005; 1:6. [PubMed: 15813993]
- Xu H, Wu LJ, Wang H, Zhang X, Vadakkan KI, Kim SS, Steenland HW, Zhuo M. Presynaptic and postsynaptic amplifications of neuropathic pain in the anterior cingulate cortex. *J Neurosci*. 2008; 28:7445–7453. [PubMed: 18632948]

Figure 1. Placements for nicotine and DHβE infusions. A) Anterior Cingulate - nicotine; B) Anterior Cingulate - DHβE with nicotine and ethanol; C) Anterior Cingulate - DHβE with ethanol D) Dorsal Hippocampus; E) Ventral Hippocampus (Numbers indicate anterior/posterior mm from Bregma).

Figure 2.

Interactive effects of ethanol and nicotine infused into the anterior cingulate in the PMDAT. (A–B) Effects on learning [percent time in the aversive arm vs. non-aversive arm]: A) Ethanol (1.4 g/kg), nicotine, and nicotine plus ethanol (1.0–1.4 g/kg) decreased time in the non-aversive arm compared to controls at training. B) Ethanol (1.0–1.4 g/kg) administered at training increased time in the aversive arm and decreased time in the non-aversive at testing, and nicotine administered at training reversed these effects ($n=6-8$; Mean $\pm$ SEM; * = significantly different from controls at $p<0.05$; += significant difference between arms). (C–D) Effects on anxiety [percent time in the open arms]: C) Ethanol (1.4 g/kg) and nicotine alone increased time in the open arms at training. D) Ethanol (1.4 g/kg) administered at training increased time in the open arms at testing, and nicotine failed to alter this effect ($n=6-8$; Mean $\pm$ SEM; * = significantly different from controls at $p<0.05$). (E–F) Effects on locomotion [arm entries per minute]: (E) Ethanol (1.4 g/kg) increased locomotion at training and nicotine administered reversed this effect. (F) Ethanol (1.4 g/kg) administered at training increased locomotion at testing, and nicotine administered at training reversed this effect ($n = 6-8$; Mean $\pm$ SEM; * = significantly different from controls at $p<0.05$).

Figure 3.

Interactive effects of systemic ethanol and nicotine and DH β E infused into the anterior cingulate in the PMDAT. (A–B) Effects on learning [percent time in the aversive arm vs. non-aversive arm]: A) Ethanol plus nicotine decreased time in the non-aversive arm compared to controls at training and DH β E failed to alter this effect. B) Ethanol administered at training increased time in the aversive arm and decreased time in the non-aversive arm at testing, nicotine administered at training reversed these effects, and DH β E blocked the effect of nicotine (n=6–8; Mean $\pm$ SEM; * = significantly different from controls at p<0.05; + = significant difference between arms). (C–D) Effects on anxiety [percent time in the open arms]: C) Ethanol plus nicotine and DH β E increased time in the open arms at training. D) There were no effects on anxiety at testing (n=6–8; Mean $\pm$ SEM; * = significantly different from controls at p<0.05). (E–F) Effects on locomotion [arm entries per minute]: There were no effects on locomotion at training (E) or testing (F) (n = 6–8; Mean $\pm$ SEM; * = significantly different from controls at p<0.05).

Figure 4.

Interactive effects of systemic ethanol and DHβE infused into the anterior cingulate in the PMDAT. (A–B) Effects on learning [percent time in the aversive arm vs. non-aversive arm]: A) There were no significant differences between groups at training. B) Ethanol administered at training increased time in the aversive arm and decreased time in the non-aversive arm at testing, and DHβE reversed these effects ($n=6-8$; Mean $\pm$ SEM; * = significantly different from controls at $p<0.05$; + = significant difference between arms). (C–D) Effects on anxiety [percent time in the open arms]: There were no effects on anxiety at training (C) or testing (D) ($n=6-8$; Mean $\pm$ SEM; * = significantly different from controls at $p<0.05$). (E–F) Effects on locomotion [arm entries per minute]: There were no effects on locomotion at training (E) or testing (F) ($n=6-8$; Mean $\pm$ SEM; * = significantly different from controls at $p<0.05$).

Figure 5.

Interactive effects of ethanol and nicotine infused into the dorsal hippocampus in the PMDAT. (A–B) Effects on learning [percent time in the aversive arm vs. non-aversive arm]: A) Ethanol (1.4 g/kg) decreased time in the non-aversive arm compared to controls at training. B) Ethanol (1.0–1.4 g/kg) administered at training increased time in the aversive arm at testing, and nicotine administered at training failed to reverse this effect ($n=6-8$; Mean $\pm$ SEM; * = significantly different from controls at $p<0.05$; += significant difference between arms). (C–D) Effects on anxiety [percent time in the open arms]: C) Ethanol (1.4 g/kg) increased time in the open arms at training, and nicotine blocked this effect. D) Ethanol (1.4 g/kg) administered at training also increased time in the open arms at testing, and nicotine administered at training blocked this effect ($n=6-8$; Mean $\pm$ SEM; * = significantly different from controls at $p<0.05$). (E–F) Effects on locomotion [arm entries per minute]: (E) Ethanol (1.4 g/kg) increased locomotion at training and nicotine reversed this effect. (F) Ethanol (1.0–1.4 g/kg) administered at training increased locomotion at testing, and nicotine administered at training partially ameliorated this effect ($n=6-8$; Mean $\pm$ SEM; * = significantly different from controls at $p<0.05$).

Figure 6.

Interactive effects of ethanol and nicotine infused into the ventral hippocampus in the PMDAT. (A–B) Effects on learning [percent time in the aversive arm vs. non-aversive arm]: A) Ethanol (1.4 g/kg) and nicotine plus ethanol (1.0–1.4 g/kg) decreased time in the non-aversive arm compared to time in the aversive arm at training. B) Ethanol (1.0–1.4 g/kg) administered at training increased time in the aversive arm and decreased time in the non-aversive arm at testing, and nicotine administered at training failed to reverse this effect ($n=6-8$; Mean $\pm$ SEM; * = significantly different from controls at $p<0.05$; += significant difference between arms). (C–D) Effects on anxiety [percent time in the open arms]: C) Ethanol (1.4 g/kg) and nicotine plus ethanol (1.0–1.4 g/kg) increased time in the open arms at training. D) Neither drug altered anxiety at testing ($n=6-8$; Mean $\pm$ SEM; * = significantly different from controls at $p<0.05$). (E–F) Effects on locomotion [arm entries per minute]: There were no effects on locomotion at training (E) or testing (F) ($n = 6-8$; Mean $\pm$ SEM).