

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

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The current study examined the effects of nicotine infusion into the dorsal hippocampus or anterior cingulate on fear conditioning and on ethanol-induced deficits in fear conditioning, and whether these effects involved receptor activation or inactivation. Conditioning consisted of two white noise (30 s, 85 dB)–foot-shock (2 s, 0.57 mA) pairings. Saline or ethanol was administered to C57BL/6 mice 15 min before training and saline or nicotine was administered 5 min before training or before training and testing. The ability of the high-affinity nicotinic acetylcholinergic receptor (nAChR) antagonist dihydro- β -erythroidine (DH β E) to modulate the effects of ethanol and nicotine was also tested; saline or DH β E was administered 25 (injection) or 15 (infusion) minutes before training or before training and testing. Infusion of nicotine into the hippocampus enhanced contextual fear conditioning but had no effect on ethanol-induced learning deficits. Infusion of nicotine into the anterior cingulate ameliorated ethanol-induced deficits in contextual and cued fear conditioning but had no effect on learning in ethanol-naïve mice. DH β E blocked the effects of nicotine on ethanol-induced deficits; interestingly, DH β E alone and co-administration of subthreshold doses of DH β E and nicotine also ameliorated ethanol-induced deficits but failed to enhance learning. Finally, DH β E failed to ameliorate ethanol-induced deficits in β 2 nAChR subunit knockout mice. These results suggest that nicotine acts in the hippocampus to enhance contextual learning, but acts in the cingulate to ameliorate ethanol-induced learning deficits through inactivation of high-affinity β 2 subunit-containing nAChRs.

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INTRODUCTION

Despite the well-known consequences of alcohol and nicotine consumption, abuse and co-abuse of these drugs remain a major societal issue. A positive correlation exists between high-risk, heavy use of each drug (Dawson, 2000; John *et al*, 2003; Larsson and Engel, 2004). There is also a correlation between dependence on alcohol and dependence on nicotine; 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 population to smoke cigarettes.

Although alcohol and nicotine have anxiolytic (Blanchard *et al*, 1993; Picciotto *et al*, 2002) and rewarding (White,

1996) effects, the co-abuse of these drugs may also result from the interaction of these drugs on cognitive function. For instance, ethanol disrupts contextual and cued fear conditioning (Escher and Mittleman, 2004; Gould, 2003; Gulick and Gould, 2007), whereas nicotine enhances contextual but not cued fear conditioning (Gould and Higgins, 2003a; Gould and Wehner, 1999) and ameliorates ethanol-induced deficits in both contextual and cued fear conditioning (Gould and Lommock, 2003b; Gulick and Gould, 2008). However, it is unclear whether the same mechanism involved in the enhancement of contextual fear conditioning by nicotine mediates nicotine amelioration of ethanol-induced deficits in fear conditioning.

Although nicotine infusion into the dorsal hippocampus enhances contextual fear conditioning (Davis *et al*, 2007), it is unknown if nicotine acts in this area to ameliorate ethanol-induced learning deficits. Nicotine modulates ethanol-induced deficits in both cued and contextual fear conditioning. Cued fear conditioning, however, is hippocampus-independent (Logue *et al*, 1997; Phillips and LeDoux, 1992); thus, nicotine may act in another brain

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area to ameliorate ethanol-induced deficits in contextual and cued fear conditioning. The anterior cingulate is critically involved in attentional processes (Allman *et al*, 2001), and mnemonic processes (Chiba *et al*, 1997; Faw, 2003; Malin *et al*, 2007; Malin and McGaugh, 2006; Tang *et al*, 2005). With roles in both attention and learning, the anterior cingulate represents an area where nicotine may act to alter ethanol-induced deficits in fear conditioning.

Just as the brain areas involved in the interactive effects of ethanol and nicotine on learning are unknown, so are the underlying receptor-level processes. Neuronal nicotinic acetylcholinergic receptors (nAChRs) are a broad family of pentameric ion channels comprised of either α ($\alpha 2-10$) or α and β ($\beta 2-4$) subunits (Decker *et al*, 1995). $\beta 2$ -containing nAChRs are involved in both the nicotine enhancement of learning (Davis *et al*, 2007) and the interactive effects of nicotine and ethanol on learning (Wehner *et al*, 2004). Furthermore, $\alpha 4\beta 2^*$ (* denotes potential unknown additional subunits) nAChRs are found in the dorsal hippocampus and anterior cingulate cortex (Alkondon and Albuquerque, 1993; Pichika *et al*, 2006). However, nicotine can both activate and desensitize nAChRs (for review, Picciotto *et al*, 2008); thus, nAChR activation and inactivation may differentially modulate the effects of nicotine on learning and on ethanol-induced deficits in learning.

In the current study, we compared the effects of nicotine infusion into the dorsal hippocampus and anterior cingulate on learning and on ethanol-induced deficits in learning. We also compared the effects of cingulate infusion of nicotine versus dihydro- β -erythroidine (DH β E, a high-affinity nAChR antagonist) on ethanol-induced learning deficits to determine whether receptor activation or inactivation can ameliorate these deficits. Because DH β E antagonizes multiple high-affinity nAChRs (Harvey *et al*, 1996; Williams and Robinson, 1984), $\beta 2$ nAChR subunit knockout (KO) mice were used to test if the effects of DH β E on ethanol-induced fear conditioning deficits involve $\beta 2$ -containing nAChRs.

MATERIALS AND METHODS

Subjects

Male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were tested at 8–12 weeks of age (20–30 g). Heterozygous $\beta 2$ nAChR subunit knockout (KO) mice (original breeding pairs were backcrossed to C57BL/6 mice for at least seven generations and provided by Dr Arthur Beaudet, Baylor College of Medicine) were bred to obtain male and female $\beta 2$ nAChR subunit KO mice (ages 8–12 weeks), and wild-type littermates (WT; 8–12 weeks of age); genotype was verified by PCR. Numerous studies have used the $\beta 2$ KO mice backcrossed onto a C57BL/6 background to investigate the effects of nicotine on behavior and brain function (Portugal *et al*, 2008; Raybuck and Gould, 2009; Salas *et al*, 2004; Wehner *et al*, 2004). Mice were housed in groups of four per cage before surgery and individually housed after surgery. Mice had *ad libitum* access to food and water. A 12-hr light–dark cycle (lights on at 0700 hours) was maintained, with all testing done between 0900 and 1700 hours. Procedures were approved by the Temple University Institutional Animal Care and Use Committee.

Apparatus

Training and context testing took place in identical conditioning chambers housed in sound-attenuating boxes (MED Associates, St Albans, VT). Each $17.78 \times 19.05 \times 38.10$ cm chamber consisted of Plexiglas panels in the front, back, and ceiling and two stainless-steel walls on the sides. The metal grid floor of each chamber, through which the foot-shock unconditioned stimulus (US; 0.57 mA for 2 s) was delivered, was connected to a shock generator and scrambler. Speakers that delivered a white noise conditioned stimulus (CS; 85 dB for 30 s) were mounted on the right wall of each chamber. Background noise (69 dB) and air exchange were provided by ventilation fans mounted on the right wall of each sound-attenuating box. A computer running MED Associates software controlled stimuli presentation. Chambers were cleaned with 70% ethanol before each session.

Testing for freezing to the CS occurred in a separate room in altered context chambers housed in sound-attenuating boxes. Speakers that delivered the white noise CS were mounted on the left wall of each chamber. The $20.32 \times 22.86 \times 17.78$ cm chambers were constructed of four Plexiglas walls, a Plexiglas ceiling, and an opaque white plastic floor. In addition to the differences in location, visual cues (eg, the inside of the sound-attenuating boxes in the training context were white versus black in the altered context room), chamber dimensions, floor construction, and a vanilla extract olfactory cue (no such cue was present at training) further distinguished the altered context chambers from the original training chambers.

Fear Conditioning

At training, baseline freezing was recorded for the first 120 s (based on Gould and Higgins, 2003a). At 120 and 270 s, the CS sounded for 30 s; the US occurred during the last 2 s of the CS; immediate freezing was measured during the inter-trial interval. The mice remained in the chamber for 30 s after the second CS–US presentation. Twenty-four hours later, freezing to the context was assessed for 5 min and 1 h later, generalized freezing to the altered context test was measured for 3 min (pre-CS test) followed by a 3 min test of freezing to the CS (CS test). Freezing, defined as the absence of visible movement with the exception of respiration, was assessed at 10 s intervals for 1 s.

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 determined from bregma using the mouse brain atlas (Paxinos and Franklin, 2001) were as follows: Hippocampus: -1.7 mm posterior; ± 1.5 mm mediolateral; -2.3 mm ventral; Cingulate: $+2.0$ mm anterior; ± 1.5 mm mediolateral (± 2.5 mm mediolateral for lateral controls); -2.3 mm ventral (-4.0 mm ventral for

below controls); it should be noted that in rats, functional differences in rostral (2.6 AP rats) and caudal (0.2 AP rats) anterior cingulate cortex (Johansen *et al*, 2001; Malin *et al*, 2007) exist. The mouse cingulate cortex extends from 2.34 AP to -0.22 AP, and then continues to -0.82 as the cingulate/retrosplenial complex. All of our cingulate infusions were restricted to a rostral range of 2.34 to 1.70 AP. Ketoprofen (2.0 mg/kg) was administered subcutaneously for post-operative pain. Animals were allowed at least 5 days to recover before behavioral procedures began.

Drugs and Infusion

Nicotine hydrogen tartrate salt (Sigma St. Louis, MO; 0.045 or 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 *et al* (2007) and Davis and Gould (2006)) and DH β E (Sigma St. Louis, MO; 3.0 or 6.0 mg/kg subcutaneous (s.c) injection or 18.0 μ g/0.50 μ l/side infusion based on Davis *et al* (2007) and Davis and Gould (2006)) were dissolved in physiological saline; we were unable to test the effects of higher systemic doses of DH β E as doses of 8.0 mg/kg or higher induced motor depression. Injection volume for nicotine and DH β E was 0.01 ml/g body weight, and for ethanol (Fisher Scientific Pittsburgh, PA; 1.0 g/kg i.p. based on Gould, 2003) it was 20% vol/vol in saline. Controls received physiological saline. DH β E was infused (15 min) or injected (25 min) before training or before training and testing, ethanol was injected 15 min before training, and nicotine was infused or injected 5 min before training or before training and testing. Injection and infusion times were based on previous research (Davis and Gould, 2006; Davis *et al*, 2007; Gulick and Gould, 2008).

For direct infusions, mice were gently restrained and dummy cannulae were removed and replaced with 22-G infusion cannulae. Drugs were bilaterally infused at a rate of 0.50 μ l/min. 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 min after infusion to allow drug diffusion away from the cannula tip.

Histology

Brains were placed in a 10% formalin solution (Fisher Scientific, Pittsburgh, PA) for at least 24 h before 60 μ m thick coronal sections were sliced at -18°C . The sections were stained with cresyl violet and cannula placements were determined using a light microscope. Data from animals with incorrect placements were excluded from statistical analysis (<10%).

Experimental Design

We first tested the effects of nicotine infusion into the dorsal hippocampus or anterior cingulate at both training and testing on systemic ethanol-induced fear conditioning deficits. A second experiment examined whether nicotine administration at training only would be sufficient to ameliorate ethanol-induced cognitive deficits; the effects of

systemic nicotine administration were tested first, followed by a test of the effects of direct infusion of nicotine into the cingulate cortex. To ensure that the effect of nicotine was specific to the anterior cingulate, we infused nicotine below and lateral to the anterior cingulate infusion site.

To determine if DH β E alters nicotine amelioration of ethanol-induced learning deficits, we systemically administered ethanol, nicotine, and DH β E before training. A follow-up experiment determined whether infusion of DH β E into the anterior cingulate cortex would alter the effects of systemic nicotine and ethanol on fear conditioning. Finally, we examined whether the effects of DH β E on ethanol-induced learning deficits involve β 2-containing nAChRs by systemically administering the drugs to β 2 KO and WT mice.

Shock Sensitivity

We next determined whether the interactive effects of ethanol and nicotine on fear conditioning were mediated by changes in shock sensitivity. Animals were systemically administered either saline, 1.0 g/kg ethanol (10 min before testing), or 0.09 mg/kg nicotine (immediately before testing), and then exposed to a range of 2 s foot shocks (0.10–0.60 mA) over a 15 min testing period. There were three presentations at each shock intensity, with a 20 s inter-stimulus interval and a 90 s inter-trial interval. Each animal was administered all three drug treatments, one on each of three consecutive days, and testing sessions occurred 24 h apart with counterbalancing of the order of drug administration. We scored vocalization (yes or no) and motion (0 = no response; 1 = hop; 2 = jump; 3 = run; 4 = horizontal jump; 5 = vertical jump) for each shock presentation (scoring based on Bardgett *et al*, 2003; Schrott and Crnic, 1994).

Statistical Testing

Data were analyzed using a repeated-measures or two-way analysis of variance (ANOVA), or independent samples *t*-test in the DH β E-nicotine-alone studies. Tukey's *post hoc* analysis was used to detect significant differences at $P < 0.05$ (SPSS version 13; Chicago, IL).

RESULTS

Nicotine Infusion into the Dorsal Hippocampus

The ability of nicotine infusion into the dorsal hippocampus at training and testing, alone or concurrent with training day systemic ethanol administration, to alter contextual and cued fear conditioning was examined. The levels of baseline freezing, measured before the first CS presentation, and immediate freezing, measured after the first CS-US presentation, were similar across all groups. Significant effects of ethanol, $F(1,27) = 99.17$, $P < 0.001$, and nicotine, $F(1,27) = 6.59$, $P < 0.05$, on freezing to the context on testing day were found, but there was no interactive effect of ethanol and nicotine. There was also a significant effect of ethanol on freezing to the CS, $F(1,27) = 45.67$, $P < 0.001$, but there was no effect of nicotine on freezing to the CS nor was there a significant ethanol by nicotine interaction. There

were no significant differences in freezing during the pre-CS period (Figure 1).

Post hoc analysis revealed that during context testing, the ethanol-treated groups ($n=7$ for each) froze significantly less than saline controls ($n=7$) and less than the nicotine alone group ($n=8$) ($P<0.001$). The nicotine alone group froze significantly more to the context than saline controls ($P<0.05$). For cued fear conditioning, both groups administered systemic ethanol froze significantly less than the saline or the nicotine alone group ($P<0.001$); no significant difference existed between the nicotine alone group and the saline controls. Thus, nicotine infusion into the dorsal hippocampus enhanced contextual fear conditioning, but failed to ameliorate ethanol-induced learning deficits.

Nicotine Infusion into The Anterior Cingulate

The ability of nicotine infusion into the anterior cingulate at training and testing, alone or concurrent with training day systemic ethanol administration, to alter contextual and cued fear conditioning was examined next. The levels of baseline and immediate freezing were similar across all groups. Significant effects of ethanol, $F(1,35)=41.61$, $P<0.001$, and of nicotine, $F(1,35)=9.21$, $P<0.01$, on freezing to the context on testing day were found, as well as an interaction of ethanol and nicotine $F(1,35)=18.57$, $P<0.001$. There were also significant effects of ethanol, $F(1,35)=31.32$, $P<0.001$, and of nicotine, $F(1,35)=9.50$, $P<0.01$, on freezing to the CS, and an interaction of ethanol and nicotine $F(1,35)=8.58$, $P<0.01$. There were no significant differences in freezing during the pre-CS period (Figure 2).

Post hoc analysis revealed that during context testing, the ethanol-alone group ($n=10$) froze significantly less than saline controls ($n=8$) and less than the nicotine infusion groups ($P<0.001$). The nicotine alone ($n=8$) and nicotine and ethanol ($n=9$) groups were similar to saline controls. The same pattern emerged for cued fear conditioning. Thus, nicotine infusion into the anterior cingulate did not

enhance contextual fear conditioning, but ameliorated ethanol-induced deficits in contextual and cued fear conditioning.

Nicotine Administration on Training Day Only

In the previous studies, nicotine was administered at both training and testing. To test if training day administration of nicotine is sufficient to ameliorate ethanol-induced deficits, nicotine and ethanol were both administered systemically on training day only. Baseline and immediate freezing were similar across all groups. Significant effects of ethanol, $F(1,36)=215.01$, $P<0.001$, and of nicotine, $F(1,36)=8.16$, $P<0.01$, on freezing to the context on testing day were found, as well as an interaction of ethanol and nicotine $F(1,36)=4.39$, $P<0.05$. There were also significant effects of ethanol, $F(1,36)=26.15$, $P<0.001$, and of nicotine, $F(1,36)=4.39$, $P<0.05$, on freezing to the CS, as well as an interaction of ethanol and nicotine $F(1,36)=5.54$, $P<0.05$. There were no significant differences in freezing during the pre-CS period.

Post hoc analysis revealed that for context testing, both ethanol-treated groups froze significantly less than saline controls and the nicotine alone group ($P<0.001$), but the group administered ethanol and nicotine also froze significantly more than the group administered ethanol alone ($P<0.01$). The group administered nicotine alone froze at levels similar to saline controls. For cued fear conditioning, the group administered ethanol alone froze significantly less than all other groups ($P<0.01$). Nicotine groups were not significantly different from saline controls. Thus, systemic nicotine administration on training day only did not enhance contextual fear conditioning, as has previously been shown (Gould and Higgins, 2003a; Gould and Wehner, 1999); but ameliorated ethanol-induced deficits in contextual and cued fear conditioning (Table 1).

Based on the results with systemic nicotine, the effects of nicotine infusion into the anterior cingulate on training day only were assessed. Baseline and immediate freezing were

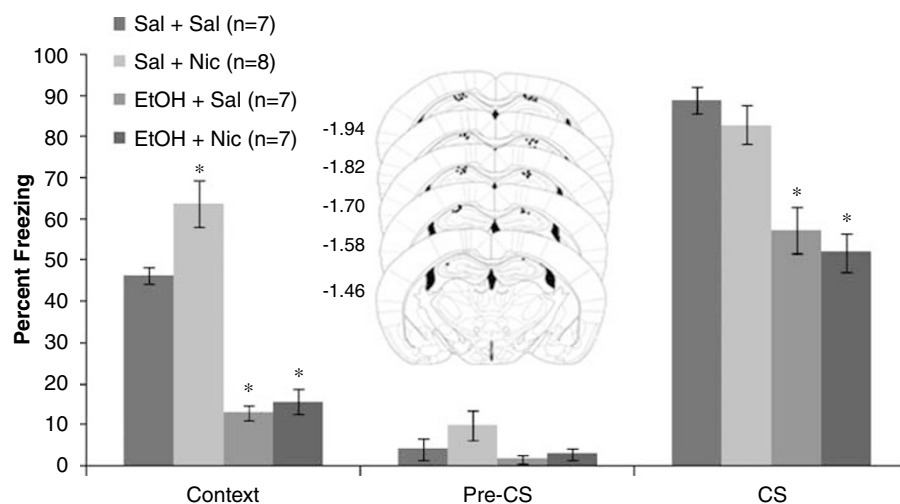


Figure 1 Bilateral nicotine infusion ($0.35 \mu\text{g}/0.50 \mu\text{l}$ per side) into the dorsal hippocampus at training and testing enhanced contextual fear conditioning but failed to ameliorate systemic ethanol-induced (1.0 g/kg) impairments in contextual and cued fear conditioning (mean \pm SEM; * indicates significant difference from controls, $P<0.05$). Center figure represents drug infusion sites.

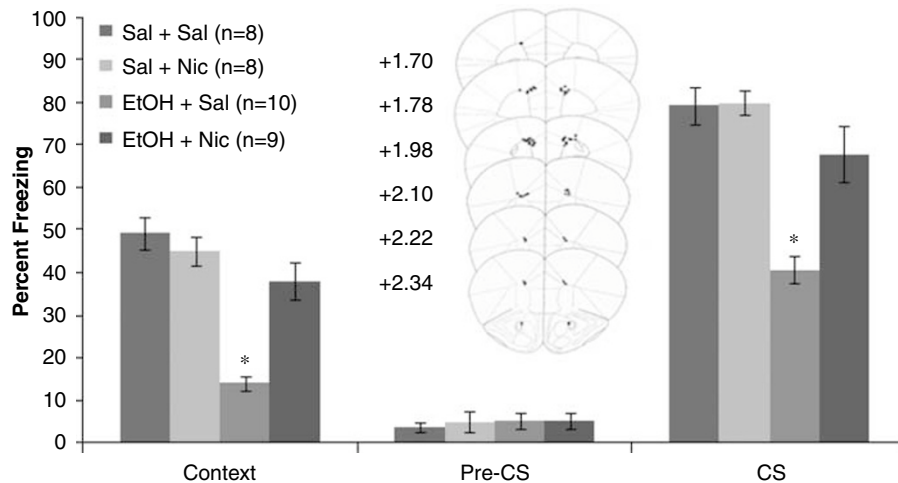


Figure 2 Bilateral nicotine infusion (0.35 $\mu\text{g}/0.50\ \mu\text{l}$ per side) into the anterior cingulate at training and testing ameliorated systemic ethanol-induced (1.0 g/kg) impairments in contextual and cued fear conditioning, but failed to enhance contextual fear conditioning (mean \pm SEM; * indicates significant difference from controls, $P < 0.05$). Center figure represents drug infusion sites.

Table 1 Systemic Nicotine on Training Day Only (mean \pm SEM)

Group	Saline n = 10	Ethanol n = 9	Nicotine n = 10	EtOH and nicotine n = 9
Context	46.7 \pm 2.6	16.0 \pm 1.3	45.3 \pm 1.7	24.7 \pm 1.3
CS	81.2 \pm 2.6	56.1 \pm 4.4	82.2 \pm 2.9	71.7 \pm 4.6

similar across all groups. Significant effects of ethanol, $F(1,28) = 35.20$, $P < 0.001$, and of nicotine, $F(1,28) = 23.01$, $P < 0.001$, on freezing to the context on testing day were found, as well as an interaction of ethanol and nicotine $F(1,28) = 33.30$, $P < 0.001$. There were also significant effects of ethanol, $F(1,28) = 45.88$, $P < 0.001$, and of nicotine, $F(1,28) = 10.03$, $P < 0.01$, on freezing to the CS, and an interaction of ethanol and nicotine $F(1,28) = 31.04$, $P < 0.001$. There were no significant differences in freezing during the pre-CS period (Figure 3).

Post hoc analysis revealed that during context testing, the ethanol-alone group ($n = 8$) froze significantly less than saline controls ($n = 7$) and less than the groups infused with nicotine ($n = 7$ for each) ($P < 0.001$). The nicotine-infused groups froze at levels similar to saline controls. The same results were seen for cued fear conditioning. Thus, similar to systemic nicotine, nicotine infusion into the anterior cingulate on training day only did not enhance contextual fear conditioning, but ameliorated ethanol-induced deficits in contextual and cued fear conditioning.

To ensure that the effects of nicotine were specific to the anterior cingulate, and not to diffusion into nearby areas, we also infused nicotine below (accessory optic tract) and lateral (motor cortex) to the anterior cingulate. This control was not necessary for the hippocampus study as it was previously conducted in our laboratory (Davis *et al.*, 2007). For both sets of infusions, baseline and immediate freezing were similar across all groups. For the below infusions, there was a significant effect of systemic ethanol on freezing to the context, $F(1,26) = 90.62$, $P < 0.001$, and to the CS,

$F(1,26) = 49.12$, $P < 0.001$, but no other significant effects. *Post hoc* analysis revealed that during context and cued testing, both ethanol-treated groups ($n = 7$ for each) froze significantly less than saline controls ($n = 7$) and the nicotine alone group ($n = 8$) ($P < 0.001$). For the lateral infusions, there was a significant effect of ethanol on freezing to the context, $F(1,24) = 105.87$, $P < 0.001$, and to the CS, $F(1,24) = 257.12$, $P < 0.001$, on testing day, but no other significant effects. *Post hoc* analysis revealed that during context and cued testing, the ethanol-treated groups ($n = 6$ for EtOH-alone and $n = 7$ for Nic and EtOH) froze significantly less than saline controls ($n = 7$ for each) and the nicotine-alone group ($n = 7$) ($P < 0.001$) (Figure 4). Thus, nicotine administration below or lateral to the anterior cingulate on training day only did not alter contextual and cued fear conditioning.

DH β E Blockade of Nicotine Amelioration of Ethanol-Induced Deficits

To determine whether the high-affinity nAChR antagonist DH β E would alter the interactive effects of nicotine and ethanol, we administered nicotine, ethanol, and DH β E systemically. Baseline and immediate freezing were similar across all groups. A significant effect of drug treatment on freezing to the context on testing day was found, $F(3,24) = 28.35$, $P < 0.001$. There was also a significant effect of drug treatment on freezing to the CS, $F(1,24) = 11.74$, $P < 0.01$. There were no significant differences in freezing during the pre-CS period. *Post hoc* tests demonstrated that ethanol decreased freezing below saline control levels for both the contextual and cued tests ($P < 0.01$), but the group treated with ethanol and nicotine froze significantly more than the ethanol-alone group ($P < 0.05$). The group administered DH β E, ethanol, and nicotine froze at similar levels to the ethanol-alone group (Table 2).

To investigate whether the 0.09 mg/kg nicotine dose and the 6.0 mg/kg DH β E dose interact to alter learning in the absence of ethanol, we administered both drugs systemically before training. Baseline freezing and immediate

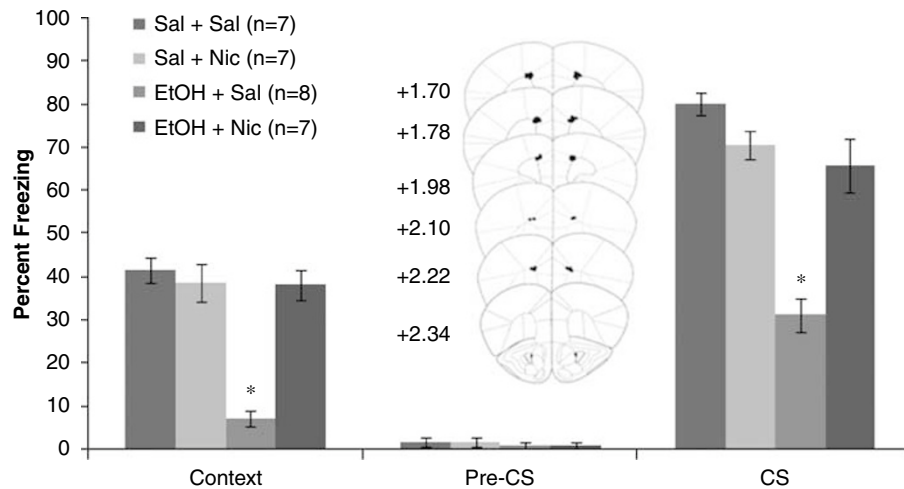


Figure 3 Bilateral nicotine infusion ($0.35 \mu\text{g}/0.50 \mu\text{l}$ per side) into the anterior cingulate on training day only ameliorated systemic ethanol-induced (1.0 g/kg) impairments in contextual and cued fear conditioning but failed to enhance contextual fear conditioning (mean \pm SEM; * indicates significant difference from controls, $P < 0.05$). Center figure represents drug infusion sites.

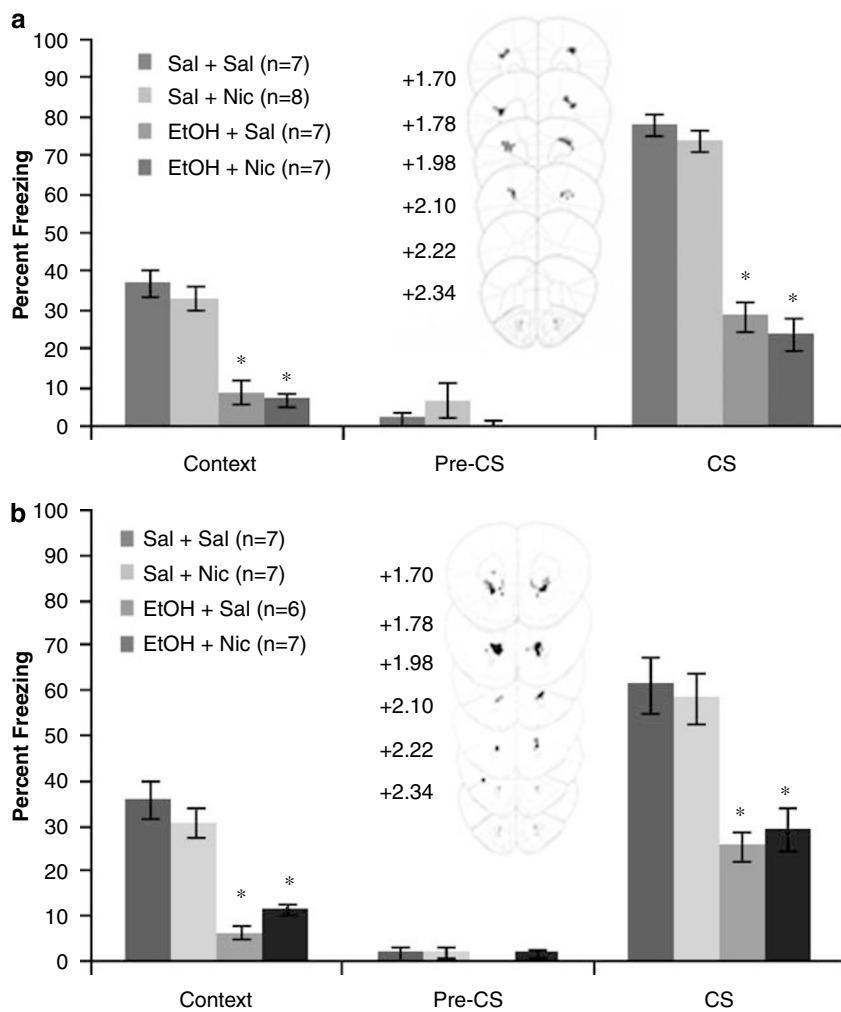


Figure 4 Bilateral nicotine infusion ($0.35 \mu\text{g}/0.50 \mu\text{l}$ per side) lateral to (a) or below (b) the anterior cingulate had no effect on contextual and cued fear conditioning or on systemic ethanol-induced deficits (mean \pm SEM; * indicates significant difference from controls, $P < 0.05$). Center figures represent drug infusion sites.

freezing were similar across all groups. Co-administration nicotine and DH β E significantly disrupted freezing to the context, $t(12) = 1.07$, $P < 0.001$, and freezing to the CS at testing, $t(12) = 0.70$, $P < 0.001$ (Table 3). There was no effect on freezing during the pre-CS period.

To examine whether the anterior cingulate is essential for the amelioration of ethanol-induced learning deficits by nicotine, we infused DH β E into the anterior cingulate and administered ethanol and nicotine systemically before training. Baseline and immediate freezing were similar across all groups. There was a significant testing day effect of drug treatment on freezing to the context, $F(3,22) = 35.40$, $P < 0.001$, and on freezing to the CS, $F(3,22) = 10.42$, $P < 0.001$. There were no significant differences in freezing during the pre-CS period (Figure 5).

Post hoc analysis revealed that the ethanol-alone ($n = 7$) and ethanol-nicotine-DH β E ($n = 9$) groups froze significantly less than saline controls ($n = 6$) and the ethanol-nicotine group ($n = 7$) to both the context ($P < 0.001$) and the CS ($P < 0.01$). The ethanol-nicotine group was not significantly different from saline controls in either test.

Table 2 Systemic DH β E, Nicotine, and Ethanol (mean \pm SEM)

Group	Saline $n = 6$	Ethanol $n = 7$	EtOH and nicotine $n = 7$	DH β E, EtOH and nicotine $n = 7$
Context	45.6 \pm 1.5	11.0 \pm 1.7	29.5 \pm 1.2	8.6 \pm 2.1
CS	89.8 \pm 2.9	31.8 \pm 4.4	76.2 \pm 5.0	41.3 \pm 4.7

Table 3 Co-administration of Systemic DH β E and Nicotine (mean \pm SEM)

Group	Saline $n = 8$	DH β E and nicotine $n = 7$
Context	32.1 \pm 2.9	7.6 \pm 1.9
CS	86.1 \pm 3.0	33.3 \pm 5.7

Thus, DH β E infused into the anterior cingulate altered the effects of systemic nicotine on ethanol-induced learning deficits. This suggests that the effects of nicotine in the anterior cingulate are both necessary and sufficient to ameliorate ethanol-induced cognitive impairments.

DH β E Amelioration of Ethanol-Induced Deficits

To determine whether DH β E may alter learning or ethanol-induced learning deficits in the absence of nicotine, we systemically administered DH β E and ethanol at training. Baseline and immediate freezing were similar across all groups. There was a significant effect of ethanol, $F(1,28) = 20.44$, $P < 0.001$, but not DH β E, on freezing to the context on testing day. There was a significant interaction of ethanol and DH β E on freezing to the context, $F(1,28) = 19.17$, $P < 0.001$. There was also a significant effect of ethanol, $F(1,28) = 32.81$, $P < 0.001$, and DH β E, $F(1,28) = 7.81$, $P < 0.05$, on freezing to the CS on testing day, as well as a significant interaction of ethanol and DH β E on freezing to the CS, $F(1,28) = 22.24$, $P < 0.001$. There were no significant differences in freezing during the pre-CS period.

Post hoc tests revealed that the ethanol-alone group froze significantly less than saline controls to both the context and the CS ($P < 0.05$). The group administered ethanol and DH β E froze significantly less than the control group to the context ($P < 0.05$), but also froze significantly more to both the context and the CS than the group administered ethanol alone ($P < 0.05$) (Table 4). Thus, systemic DH β E partially ameliorated ethanol-induced deficits in fear conditioning.

Table 4 Systemic DH β E and Ethanol (mean \pm SEM)

Group	Saline $n = 6$	Ethanol $n = 8$	DH β E $n = 7$	DH β E and EtOH $n = 8$
Context	43.3 \pm 3.0	13.8 \pm 2.2	30.5 \pm 5.1	30.0 \pm 3.4
CS	82.4 \pm 2.4	48.6 \pm 2.7	79.2 \pm 4.3	72.9 \pm 3.8

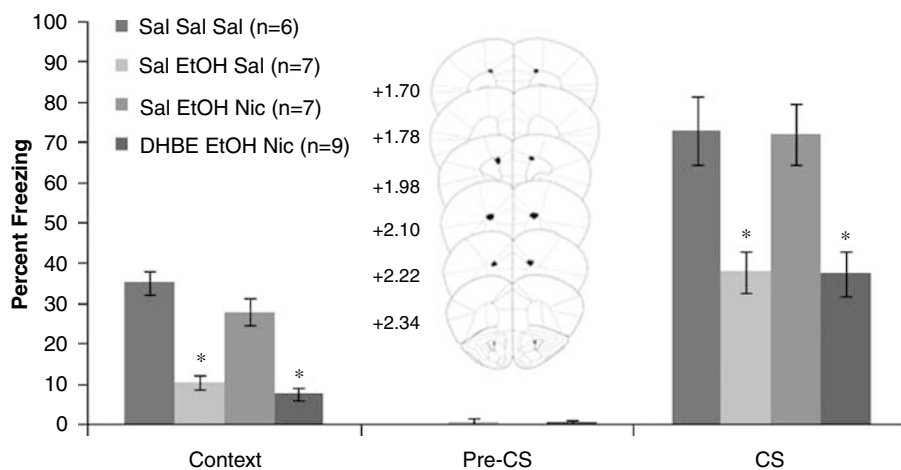


Figure 5 Bilateral DH β E infusion (18.0 μ g/0.50 μ l per side) into the anterior cingulate altered the effects of systemic nicotine (0.09 mg/kg) on systemic ethanol-induced (1.0 g/kg) impairments in contextual and cued fear conditioning (mean \pm SEM; * indicates significant difference from controls, $P < 0.05$). Center figure represents drug infusion sites.

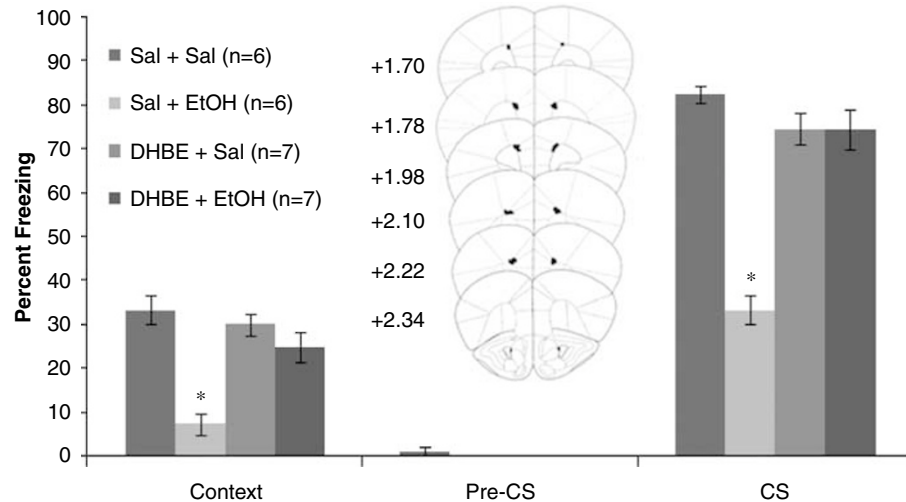


Figure 6 Bilateral DH β E infusion (18.0 μ g/0.50 μ l per side) into the anterior cingulate ameliorated systemic ethanol-induced (1.0 g/kg) impairments in contextual and cued fear conditioning but did not alter contextual or cued fear conditioning when administered alone (mean \pm SEM; * indicates significant difference from controls, $P < 0.05$).

We next examined whether DH β E infused into the anterior cingulate alters ethanol-induced learning deficits. Baseline and immediate freezing were similar across all groups. There was a significant effect of ethanol, $F(1,25) = 33.25$, $P < 0.001$, but not DH β E, on freezing to the context on testing day, and a significant interaction of ethanol and DH β E, $F(1,25) = 14.74$, $P < 0.001$. There was also a significant effect of ethanol, $F(1,25) = 55.17$, $P < 0.001$, and DH β E, $F(1,25) = 25.67$, $P < 0.05$, on freezing to the CS on testing day, and a significant interaction of ethanol and DH β E, $F(1,25) = 15.17$, $P < 0.001$. No significant differences in freezing existed during the pre-CS period (Figure 6).

Post hoc tests revealed that the group administered systemic ethanol alone ($n = 6$) froze significantly less to the context and to the CS than saline controls ($n = 6$) ($P < 0.05$). The DH β E infusion groups ($n = 7$ each) were not significantly different from saline controls in freezing to the context or the CS. Thus, infusion of DH β E into the anterior cingulate cortex ameliorated ethanol-induced learning deficits but did not alter learning in the absence of ethanol.

Amelioration of Ethanol-Induced Deficits by Subthreshold Doses of Nicotine and DH β E

Because both nicotine and DH β E ameliorated ethanol-induced deficits, we tested if co-administration of subthreshold doses of each would act synergistically on ethanol-induced deficits. We first determined that a twofold dilution of our effective doses of nicotine (0.045 mg/kg) and DH β E (3.0 mg/kg) would not ameliorate ethanol-induced deficits. For nicotine, baseline and immediate freezing were similar across all groups. At context testing, there was only a significant effect of ethanol, $F(1,27) = 196.68$, $P < 0.001$. There was a significant effect of ethanol, $F(1,27) = 400.34$, $P < 0.001$, and nicotine, $F(1,27) = 8.34$, $P < 0.01$, on freezing to the CS, but no interaction. There were no significant differences in freezing during the pre-CS period. *Post hoc* tests revealed that both groups administered ethanol froze significantly less to both the context and the CS than saline controls ($P < 0.05$), whereas the group administered nico-

Table 5 Ethanol and Subthreshold Doses of Systemic Nicotine or DH β E (mean \pm SEM)

Group	Saline $n = 7$	Ethanol $n = 7$	Nicotine $n = 7$	EtOH and nicotine $n = 7$
Context	44.3 \pm 2.5	14.3 \pm 2.2	39.1 \pm 2.4	12.4 \pm 1.5
CS	81.7 \pm 2.5	38.9 \pm 3.2	89.5 \pm 2.6	44.4 \pm 1.3
Group	Saline $n = 8$	Ethanol $n = 8$	DH β E $n = 8$	EtOH and DH β E $n = 9$
CS	45.0 \pm 2.8	16.3 \pm 2.3	29.1 \pm 2.6	13.7 \pm 1.6
Group	85.4 \pm 1.6	54.9 \pm 4.8	65.6 \pm 7.0	47.5 \pm 3.4

tine alone was not significantly different from saline controls (Table 5). Thus, 0.045 mg/kg nicotine did not ameliorate ethanol-induced learning deficits.

For the 3.0 mg/kg DH β E dose, baseline and immediate freezing were similar across all groups. There was a significant effect of ethanol, $F(1,29) = 95.69$, $P < 0.001$, and DH β E, $F(1,29) = 22.34$, $P < 0.01$, on freezing to the context on testing day, and a significant interaction, $F(1,29) = 12.60$, $P < 0.05$. There was a significant effect of ethanol, $F(1,29) = 30.67$, $P < 0.01$, and DH β E, $F(1,29) = 10.73$, $P < 0.01$, on freezing to the CS, but no significant interaction. There were no significant differences in freezing during the pre-CS period. *Post hoc* tests revealed that both ethanol groups froze significantly less to both the context and the CS than saline controls ($P < 0.05$), as did the group administered DH β E alone (Table 5). Thus, 3.0 mg/kg DH β E did not ameliorate ethanol-induced learning deficits.

We next co-administered the subthreshold, systemic doses of both drugs with ethanol at training. Baseline and immediate freezing were similar across all groups. On testing day, there was a significant effect of drug treatment on freezing to the context, $F(2,19) = 30.34$, $P < 0.001$, and the CS, $F(2,19) = 27.54$, $P < 0.001$. There were no significant differences in freezing during the pre-CS period (Figure 7).

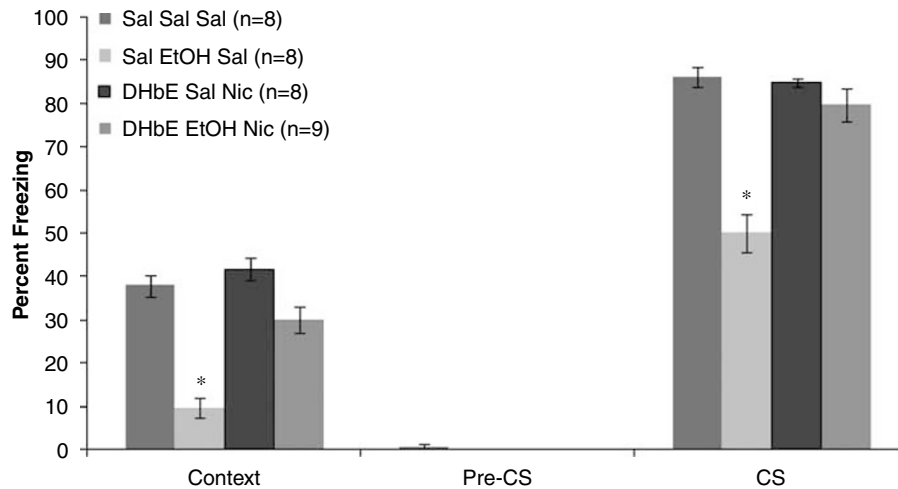


Figure 7 Co-administration of subthreshold systemic doses of DHβE (3.0 mg/kg) and nicotine (0.045 mg/kg) ameliorated systemic ethanol-induced (1.0 g/kg) impairments in contextual and cued fear conditioning but did not alter conditioning in the absence of ethanol (mean ± SEM; * indicates significant difference from controls, $P < 0.05$).

Post hoc analysis revealed that only the ethanol-alone group ($n = 8$) froze significantly less than saline controls ($n = 8$) to the context and to the CS ($P < 0.001$). The group administered DHβE, ethanol, and nicotine ($n = 9$) and the group administered DHβE and nicotine ($n = 8$) were not significantly different from saline controls. Thus, co-administration of subthreshold doses of DHβE and nicotine ameliorated ethanol-induced learning deficits.

To determine whether co-administration of systemic subthreshold doses of nicotine and DHβE could enhance learning in the absence of ethanol, mimicking the effect of nicotine on contextual fear conditioning, DHβE and nicotine were administered before training and testing. Baseline and immediate freezing were similar across all groups. There was no significant drug effect on freezing to the context on testing day, but a significant effect of drug treatment on freezing to the CS on testing day, $t(14) = 3.04$, $P < 0.05$; the group administered nicotine and DHβE froze significantly less than the saline group to the CS (Table 6). Thus, co-administration of subthreshold doses of DHβE and nicotine do not enhance learning, suggesting that the enhancement of contextual fear conditioning and the amelioration of ethanol-induced deficits in contextual and cued fear conditioning by nicotine occur through different processes.

DHβE Fails to Ameliorate Ethanol-Induced Deficits in β2 Knockout Mice

To determine whether β2-containing receptors are involved in the DHβE amelioration of ethanol-induced learning deficits, we co-administered systemic DHβE and ethanol to β2 KO and WT mice. Baseline and immediate freezing were similar across all groups. There was a significant effect of drug, $F(2,34) = 49.17$, $P < 0.001$, and genotype, $F(1,35) = 20.59$, $P < 0.001$, and a significant interaction on freezing to the context on testing day, $F(2,34) = 17.98$, $P < 0.001$. For freezing to the CS on testing day, there was a significant effect of drug, $F(2,34) = 45.41$, $P < 0.001$, and genotype, $F(1,35) = 19.72$, $P < 0.001$, and a significant

Table 6 Co-administration of Systemic Subthreshold Doses of Nicotine and DHβE (mean ± SEM)

Group	Saline $n = 8$	DHβE and nicotine $n = 8$
Context	32.3 ± 3.2	27.1 ± 1.7
CS	90.3 ± 2.7	79.9 ± 2.5

interaction, $F(2,34) = 10.70$, $P < 0.001$. There were no significant differences in freezing during the pre-CS period (Figure 8).

Post hoc tests revealed that the WT ($n = 7$) and KO ($n = 7$) saline controls froze at similar levels to both the context and the CS. The groups administered ethanol alone (WT, $n = 7$; KO, $n = 8$) froze significantly less than saline controls in both tests ($P < 0.05$), but there were no differences between genotypes in the effect of ethanol. In the WT mice ($n = 7$), DHβE ameliorated the ethanol-induced deficit in both tests ($P < 0.05$). No such effect was seen in the KO mice ($n = 7$). Thus, a β2-containing nAChR, such as the α4β2* receptor, is involved in the DHβE amelioration of ethanol-induced learning impairments.

Effects of Ethanol and Nicotine on Shock Sensitivity

Finally, we examined whether changes in sensitivity to the foot shock may underlie the effects of ethanol or nicotine on fear conditioning. Repeated-measures ANOVA revealed no significant effect of treatment order on motor responding or the number of vocalizations, so we collapsed across days. There were significant effects of shock intensity on motor responding, $F(7,280) = 477.75$, $P < 0.05$, and vocalization, $F(7,280) = 134.19$, $P < 0.05$, but no drug effects or interactions ($n = 12$ for all groups; Figure 9). Thus, neither ethanol nor nicotine significantly altered responding to the shock.

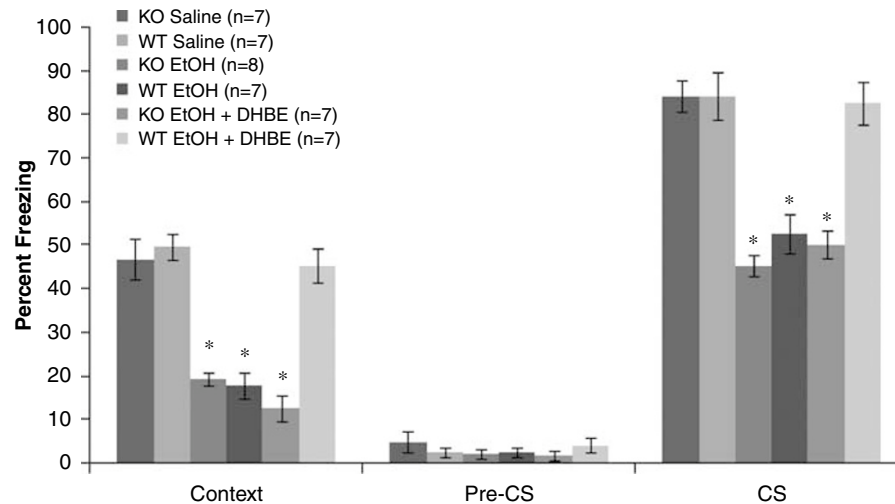


Figure 8 Systemic DH β E (6.0 mg/kg) ameliorated systemic ethanol-induced (1.0 g/kg) impairments in contextual and cued fear conditioning in wild-type mice, but failed to ameliorate these deficits in β 2-knockout mice. There were no other differences between the genotypes in their responses to the drugs (mean \pm SEM; * indicates significant difference from controls, $P < 0.05$).

DISCUSSION

Nicotine enhancement of learning and amelioration of ethanol-associated learning deficits are mediated by different neural processes. High-affinity nAChRs in the dorsal hippocampus are involved in the enhancement of contextual fear conditioning by nicotine, whereas high-affinity nAChRs in the anterior cingulate mediate the effects of nicotine on ethanol-induced deficits in contextual and cued fear conditioning. Moreover, nicotine may enhance learning by activating β 2-containing hippocampal nAChRs but ameliorate ethanol-induced learning deficits by inactivating β 2-containing anterior cingulate nAChRs.

The dorsal hippocampus processes contextual information during fear conditioning (Esclassan *et al*, 2009; Maren *et al*, 1997; Otto and Poon, 2006; Phillips and LeDoux, 1994). Previous research has demonstrated that nicotine infusion into the dorsal hippocampus enhances contextual, but not cued fear conditioning (Davis *et al*, 2007), demonstrating that the dorsal hippocampus is sufficient for the effects of nicotine on contextual fear conditioning. If nicotine is given systemically and the high-affinity nAChR antagonist DH β E is infused into the dorsal hippocampus, no enhancement of contextual fear conditioning is seen (Davis *et al*, 2007), demonstrating that the dorsal hippocampus is necessary for the enhancement of contextual fear conditioning. However, if nicotine is infused into the dorsal hippocampus of ethanol-treated mice, there is no amelioration of the ethanol-induced deficits in contextual and cued fear conditioning.

In contrast, infusion of nicotine into the anterior cingulate cortex ameliorated ethanol-induced deficits in cued and contextual fear conditioning but had no effect in ethanol-naïve mice. Furthermore, infusion of DH β E into the anterior cingulate disrupted the effects of systemic nicotine on the ethanol-induced deficits. In addition, infusion of nicotine ventral or lateral to the anterior cingulate cortex had no effect on ethanol-induced deficits in fear conditioning, and neither nicotine nor ethanol changed sensitivity to

the foot shock. Together these studies suggest that the anterior cingulate cortex is necessary and sufficient for the effects of nicotine on ethanol-induced deficits in fear conditioning.

The anterior cingulate cortex is involved in attention and cognitive control (Cohen *et al*, 1999; Posner and Rothbart, 1998; Ridderinkhof *et al*, 2004). Ethanol-induced changes in these or other cingulate-mediated processes could alter fear conditioning. In support, numerous studies have demonstrated a link between the cingulate cortex and fear conditioning. In one study, lesions of the anterior cingulate cortex did not disrupt standard cued fear conditioning but did disrupt trace fear conditioning when tested 24 h after training (Han *et al*, 2003). However, another study found that lesions of the anterior cingulate cortex disrupted cued but not contextual fear conditioning when testing occurred 24 h after training (Bissiere *et al*, 2008). In addition, the same study found that disruption of anterior cingulate cortical function through infusion of the GABA_A antagonist muscimol altered cued fear conditioning (the effects on contextual fear conditioning were not reported). Similarly, Tang *et al* (2005) reported that infusion of the NMDA receptor antagonist APV into the anterior cingulate cortex disrupted cued fear conditioning only, but infusion of muscimol disrupted both cued and contextual fear conditioning 1 and 3 days post training. Furthermore, infusion of the muscarinic cholinergic agonist oxotremorine into the anterior cingulate cortex post training enhanced foot-shock US-related learning (Malin and McGaugh, 2006). Finally, Frankland *et al* (2004) demonstrated the involvement of this area in remote (36 days old) fear conditioning memories. These results suggest that in addition to being involved in remote fear conditioning memories, the anterior cingulate cortex modulates the early phase of fear conditioning such that disruption of anterior cingulate cortical function during this stage may be detrimental to fear conditioning. Therefore, ethanol may disrupt anterior cingulate function, resulting in a deficit in fear conditioning,

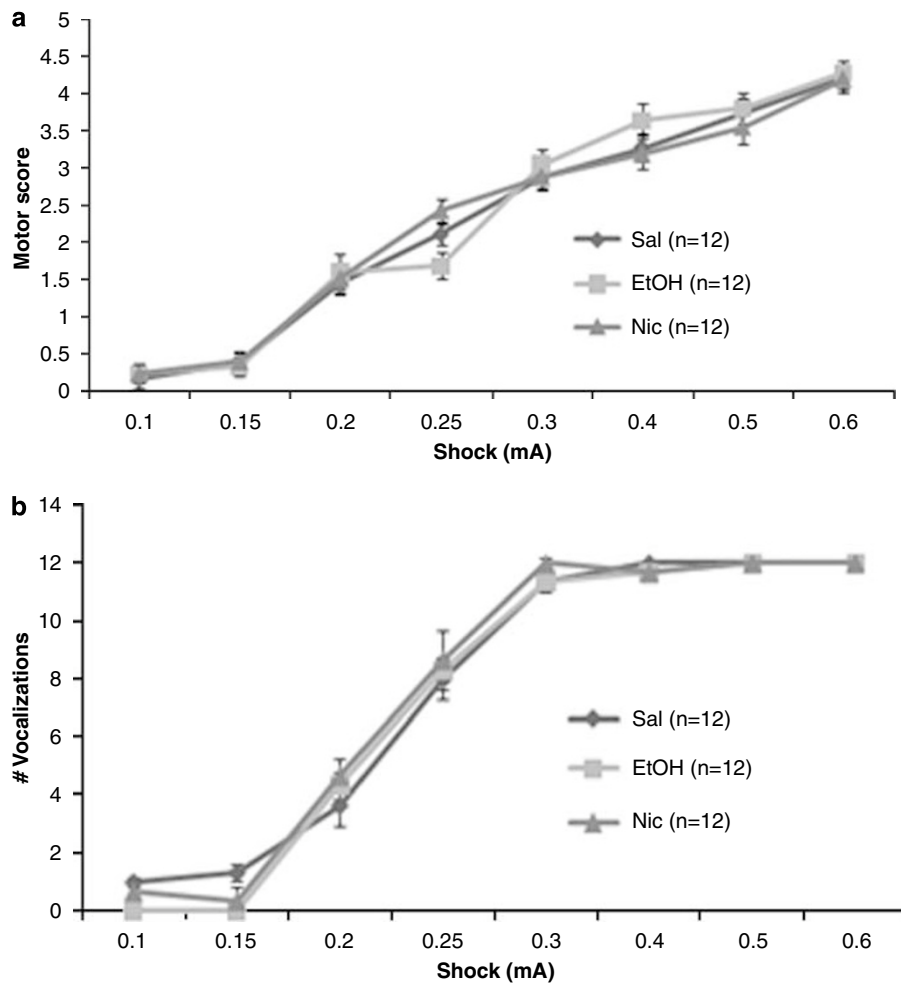


Figure 9 Effects of systemic saline, ethanol (1.0 g/kg), or nicotine (0.09 mg/kg) on shock sensitivity. There were no significant differences between groups in motor behavior (mean \pm SEM) (a) or in vocalizations (# vocalizations \pm SEM) (b) at any shock intensity.

and nicotine administration may counter this effect of ethanol.

Equally important as identifying the brain areas involved in the interactive effects of nicotine and ethanol on learning is identifying the receptor-level changes that underlie these effects. The effects of nicotine at nAChRs are complex, as nicotine can both activate and desensitize nAChRs, and these effects vary across receptor subtypes (Picciotto *et al*, 2008). $\alpha 4\beta 2^*$ nAChRs are high-affinity receptors that make up the majority of neuronal nAChRs (Whiting and Lindstrom, 1986a; Whiting and Lindstrom, 1986b). Previous work and this study suggest that nicotine enhances contextual fear conditioning by activating or activating and then desensitizing $\beta 2$ -containing nAChRs in the dorsal hippocampus (Davis and Gould, 2006), as the effects of nicotine on contextual fear conditioning are different than the effects of nAChR antagonists. $\beta 2$ -containing nAChRs also mediate the amelioration of ethanol-induced learning deficits by nicotine (Wehner *et al*, 2004), but the current findings suggest that these effects may involve nAChR inactivation. Just as nicotine ameliorated ethanol-induced deficits in fear conditioning, infusion of the nAChR antagonist DH β E into the anterior cingulate ameliorated ethanol-induced learning deficits. The effects of DH β E on

ethanol-induced deficits in fear conditioning involve $\beta 2$ -containing nAChRs as $\beta 2$ KO mice did not show DH β E amelioration of ethanol-induced learning deficits. Furthermore, co-administration of subthreshold doses of DH β E and nicotine ameliorated ethanol-induced learning deficits, suggesting that nicotine and DH β E may act synergistically on the ethanol-induced deficits. This interaction may have a dose-dependent inverted U-shaped function as co-administration of higher doses of nicotine and DH β E disrupted fear conditioning. The mechanism through which nicotine ameliorates ethanol-induced deficits in fear conditioning is still unknown but may involve the depression of nAChR activity as DH β E and nicotine had similar effects.

An important issue that needs further study is how a decrease of nAChR activity in the cingulate cortex could ameliorate ethanol-induced deficits in fear conditioning. Perhaps ethanol disrupts fear conditioning by increasing nAChR activity in the cingulate cortex. Whereas ethanol-induced upregulation of nAChR activity in the cingulate cortex has not been directly observed, it has been shown that ethanol potentiates $\alpha 4\beta 2$ -like nAChR currents in rat frontal cortical cell cultures (Aistrup *et al*, 1999; Marszalec *et al*, 1999). If ethanol is decreasing learning by

over-activating nAChRs, then a decrease in nAChR activity through nicotine desensitization of the receptors or through inhibition of the receptors by the nAChR antagonist DH β E could return the system to a level of activity optimal for learning. The finding that DH β E blocks the effects of ethanol on fear conditioning supports this theory; however, the finding that β 2 KO mice show ethanol-induced deficits in fear conditioning (Wehner *et al*, 2004 and present results) suggests this may not be the case. It is possible that an unknown developmental compensatory change in the β 2 KO mice alters the mechanism underlying ethanol effects on fear conditioning allowing ethanol to disrupt fear conditioning. Alternatively, ethanol and nicotine/DH β E may act on separate processes that have opposing actions on a common downstream target.

Interestingly, imaging studies in humans have shown that nicotine and ethanol have opposite effects on cingulate activity (Ghatan *et al*, 1998; Schreckenberger *et al*, 2004). In addition, a single nucleotide polymorphism in the gene CHRNA4 was associated with changes in attention-related activity in the anterior cingulate cortex (Winterer *et al*, 2007). This complements our finding that high-affinity nAChRs (which could include α 4 β 2* nAChRs) in the cingulate cortex are involved in the effects of nicotine on ethanol-induced deficits in fear conditioning. However, the cellular location and the processes mediated by these nAChRs remain to be determined. Picciotto *et al* (2008) put forward a model where nicotine desensitization of nAChRs on GABAergic interneurons in the ventral tegmental area underlies the behavioral effects of nicotine on reward and mood; thus, GABAergic interneurons in the cingulate cortex could similarly be involved in the effects of nicotine on ethanol-induced deficits in fear conditioning. This, however, remains to be tested as presynaptic nAChR regulation of the release of other neurotransmitters, such as dopamine, is also a viable mechanism (see Exley and Cragg, 2008 for a discussion of nAChR activation and inactivation mediating dopamine tone).

In summary, the effects of nicotine on learning in ethanol-naïve and ethanol-treated mice are mediated by different brain regions and different nAChR processes. Nicotine enhances contextual fear conditioning through either activation or activation followed by desensitization of hippocampal β 2-containing nAChR. In comparison, nicotine acts in the anterior cingulate to ameliorate ethanol-induced deficits in fear conditioning. This amelioration may depend on inactivation of β 2-containing nAChRs, as nicotine and the nAChR antagonist DH β E have similar effects on the ethanol-induced deficits. Thus, inactivation of nAChRs in the cingulate cortex could ameliorate ethanol-induced deficits in fear conditioning by countering the effects of ethanol in the cingulate cortex or in areas efferent of the cingulate cortex.

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DISCLOSURE/CONFLICT OF INTEREST

Danielle Gulick has no conflict of interest to disclose for the research reported herein. Thomas J Gould has no conflict of interest to disclose for the research reported herein.

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