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Nicotine disrupts safety learning by enhancing fear associated with a safety cue via the dorsal hippocampus

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Abstract

Learned safety, a learning process in which a cue becomes associated with the absence of threat, is disrupted in individuals with post-traumatic stress disorder (PTSD). A bi-directional relationship exists between smoking and PTSD and one potential explanation is that nicotine-associated changes in cognition facilitate PTSD emotional dysregulation by disrupting safety associations. Therefore, we investigated whether nicotine would disrupt learned safety by enhancing fear associated with a safety cue. In the present study, C57BL/6 mice were administered acute or chronic nicotine and trained over three days in a differential backward trace conditioning paradigm consisting of five trials of a forward conditioned stimulus (CS)+ (Light) coterminating with a footshock unconditioned stimulus followed by a backward CS– (Tone) presented 20 s after cessation of the unconditioned stimulus. Summation testing found that acute nicotine disrupted learned safety, but chronic nicotine had no effect. Another group of animals administered acute nicotine showed fear when presented with the backward CS (Light) alone, indicating the formation of a maladaptive fear association with the backward CS. Finally, we investigated the brain regions involved by administering nicotine directly into the dorsal hippocampus, ventral hippocampus, and prelimbic cortex. Infusion of nicotine into the dorsal hippocampus disrupted safety learning.

Keywords

Learned safety; nicotine; hippocampus; prelimbic cortex; backward conditioning

Introduction

The ability to modulate and control emotional processes is critical for normal functioning. However, in individuals with post-traumatic stress disorder (PTSD), emotional processing becomes dysregulated (Rauch et al., 2006), resulting in devastating symptoms, including re-experiencing trauma, avoidance of trauma associated cues, and increased arousal (Jovanovic

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et al., 2010; Rothbaum and Davis, 2003). Alterations in fear-related neurocognitive processes appears to be a critical component of PTSD (Shin et al., 2006). For example, individuals with PTSD present with altered anatomical and neural activation patterns in fore-brain regions important for emotional control and cognition, including the prefrontal cortex and hippocampus (Rauch et al., 2006; Shin et al., 2004, 2006). Similarly, behavioral work in human clinical populations suggests that PTSD is associated with deficits in fear inhibition (Christianson et al., 2012; Rauch et al., 2006). For example, individuals with PTSD fail to inhibit fear in the presence of cues and contexts that indicate the absence of danger (Bleichert et al., 2007; Jovanovic et al., 2009, 2010, 2012). Failure to adaptively inhibit fear is proposed to be an important factor in pathological anxiety, such as that seen in PTSD (Davis et al., 2000; Kutlu et al., 2014; Lissek et al., 2005). Thus, disruptions in fear-related learning processes, which modulate fear inhibition, may be a critical feature of PTSD symptomology.

To investigate the role of fear and fear inhibition in PTSD, Pavlovian fear conditioning, in which a neutral conditioned stimulus (CS) is paired with an aversive unconditioned stimulus (US), has been used (VanElzakker et al., 2014). For example, individuals with PTSD have deficits in fear extinction, where after fear conditioning, repeated unreinforced CS presentations result in the formation of a new “safe” association with the CS, which is expressed as a decrease in fear responding to the CS (Milad et al., 2009). However, PTSD-associated deficits in fear inhibition are not limited to previously conditioned danger cues, but are also observed in the formation of safety associations with neutral cues, that is, learned safety. Learned safety is a form of associative learning related to, but dissociable from, conditioned fear. Similar to conditioning of fear, learned safety involves presentation of an aversive US; however, the CS is presented such that it is predictive of the absence of the US. Subsequently, learned safety cues are able to elicit decreased fear in the presence of previously conditioned fear cues. Thus, learned safety has been studied as a type of conditioned inhibition (Christianson et al., 2012; Pollak et al., 2010), and learning of safety may be assessed experimentally using summation testing (Hammond, 2013). During summation testing, a fear cue (CS+) is presented in compound with a safety cue (CS–), which elicits a reduced fear response in comparison with presentation of the CS+ alone (Christianson et al., 2012; Rescorla, 1969; Williams et al., 1992). Indeed, the ability of learned safety cues to decrease fear is thought to be an adaptive learning process recruited for modulation of emotional processes (Kong et al., 2014).

A number of studies have found that PTSD is associated with deficits in learned safety (Grillon and Morgan, 1999; Jovanovic et al., 2009, 2010, 2012). For example, individuals with PTSD showed deficits in a danger/safe discrimination paradigm (Jovanovic et al., 2009, 2010), with similar fear-potentiated startle to safety and danger cues. Similarly, safety cues were found to be less effective at reducing distress in individuals with high trait anxiety (Gazendam et al., 2013). Thus, the inability to learn safety-predicting cues could play an important role in development or maintenance of PTSD symptoms.

There is a robust bidirectional association between smoking and stress and anxiety-related disorders, including PTSD (Feldner et al., 2007). Individuals with PTSD smoke more heavily and at higher rates (45% vs. 23%) compared with non-PTSD individuals (Cogle et al., 2010; Lasser et al., 2000). Conversely, prior smoking has been associated with a greater

risk of developing PTSD (Koenen et al., 2005), and individuals that reported smoking 18 months after trauma showed higher likelihood of subsequent development of anxiety symptoms and PTSD 27 months later (der Velden et al., 2007). Therefore, while it is not known how smoking facilitates or exacerbates PTSD symptoms, it appears to be a potent risk factor for development of PTSD. One possibility is that smoking alters fear learning processes that underlie PTSD anxiety-related symptoms (Kutlu and Gould, 2015). Nicotine, the psychoactive ingredient in tobacco, has strong effects on cognition in both clinical populations and pre-clinical models (Amitai and Markou, 2009; Gould et al., 2012; Kenney et al., 2012b; Levin et al., 1990; Rezvani and Levin, 2001). Importantly, acute nicotine enhanced hippocampus-dependent forms of fear learning, including trace and contextual fear conditioning (Gould et al., 2004; Gould and Wehner, 1999). In contrast, chronic nicotine does not change fear learning, suggesting the development of tolerance (Davis et al., 2005; Raybuck and Gould, 2009). Therefore, acute nicotine consumption, such as during smoking initiation, may facilitate or exacerbate PTSD symptoms by modulating hippocampus-dependent fear learning processes.

The cholinergic system is implicated in fear learning and fear inhibition (Kutlu and Gould, 2015; Wilson and Fadel, 2016), and nicotine acts directly on nicotinic acetylcholine receptors (nAChRs), ligand gated ion channels located throughout the central nervous system (Lindstrom, 1997). For example, nAChR pharmacological blockade prevents enhancement of contextual fear learning by acute nicotine (Davis and Gould, 2006). Further, activation of $\alpha 4\beta 2$ nAChRs elicits enhanced contextual fear learning (Yildirim et al., 2015). Similarly, pharmacological and genetic approaches have demonstrated that high-affinity $\alpha 4$ and $\beta 2$ containing nAChRs are important for nicotine's effects (Davis et al., 2007; Kutlu et al., 2016a). Animals lacking $\alpha 4$ or $\beta 2$ nAChR subunits show normal fear extinction when administered acute nicotine, while wildtype animals show disruptions in fear extinction (Kutlu et al., 2016a). Importantly, little is known regarding the nAChR system and danger/safe cue discrimination. One study found that acute nicotine disrupted context discrimination in a time-dependent manner, suggesting that nicotine results in discrimination deficits (Kutlu et al., 2014). In contrast, work using appetitive reinforcement found that nAChR activation enhanced cue discrimination and behavioral inhibition (MacLeod et al., 2006, 2010).

It is currently an outstanding question whether nicotine can alter learning a discrete safety cue. For example, acute nicotine has no effect on cued associative learning when the CS and US temporally overlap (Gould and Wehner, 1999), which suggests that nicotine does not intrinsically alter learning all discrete cue associations. However, acute nicotine has been shown to potentiate trace fear learning, a form of cued associative learning in which the CS and US do not temporally overlap (Gould et al., 2004). Therefore, in this study we sought to investigate the effects of acute and chronic nicotine on safety learning when the putative safety CS was presented in temporal proximity to the US. Additionally, the behavioral design of this study, in which the US and putative safety cue were separated by a temporal interval, has particular translational relevance as real-life traumatic events may occur under ambiguous conditions (Lissek et al., 2006). Moreover, the formation of inappropriate danger associations may be a critical aspect of PTSD (Grillon and Morgan, 1999).

Previous work suggests involvement of the hippocampus and medial prefrontal cortex (mPFC) in nicotine's effects on cognition and fear learning (Kenney, et al., 2012b; Raybuck and Gould, 2010). Furthermore, manipulations of the mPFC have been found to disrupt some forms of cue discrimination (Meyer and Bucci, 2014; Sangha et al., 2014) and conditioned inhibition (Rhodes and Killcross, 2007), and individuals with PTSD have altered prefrontal cortical and hippocampal activity (Rauch et al., 2006; Shin et al., 2004, 2006). Finally, learning trace associations is dependent on the mPFC and hippocampus (Connor and Gould, 2016; Gilmartin and Helmstetter, 2010; Gilmartin et al., 2013b; Misane et al., 2005; Raybuck and Gould, 2010). Therefore, we investigated the effect of nicotine administered directly into the dorsal hippocampus, ventral hippocampus, and prelimbic cortex on backward trace conditioned safety.

Methods

Subjects

For all experiments, male C57BL/6 mice, 8–12 weeks old (Jackson Laboratory, Bar Harbor, ME, USA) were used. Mice were housed in groups of four and maintained on a 12 h light/dark cycle, food and water access was ad libitum. All training and testing occurred between the hours of 09:00 and 19:00. Housing and behavioral procedures were approved by the Temple University Institutional Animal Care and Use Committee.

Apparatus

Backward trace conditioning was performed in four identical chambers contained within sound attenuating boxes (MED Associates, St. Albans, VT, USA). The sound attenuating boxes housed ventilation fans producing 69 dB background noise. The auditory CS was produced using a speaker located on the wall of the conditioning chamber. A 100 mA houselight was similarly mounted at the top of a chamber wall. The front, back and ceiling of the chamber was made of clear Plexiglas and the floor consisted of a metal grid connected to a shock generator. The shock generator produced a 2-s, 0.57 mA scrambled footshock. Testing occurred in a different room using four chambers housed within sound attenuating boxes (MED Associates, St. Albans, VT, USA). In contrast to the training chambers, the testing chambers had flat plastic floors and one wall housed an inactive nosepoke apparatus. Similar to training chambers, testing chambers housed ventilation fans that provided white noise (69 dB). For both training and testing a 6 kHz tone (85 dB) CS– was produced by a programmable audio generator (MED Associates, St. Albans, VT, USA) via speakers housed within both the training and testing chambers. Additionally, the house light was used as a CS + cue at 65 lux. Both training and testing occurred under red light conditions so as to increase salience of the light CS+. The presentation of all stimuli was controlled by Med-PC software.

Drugs

Systemic administration—The effects of acute and chronic nicotine on learned safety were examined. For all systemic studies, nicotine hydrogen tartrate (Sigma, St. Louis, MO, USA), reported as freebase weight, was dissolved in 0.9% sterile saline. To investigate the effects of acute nicotine on backward trace conditioned safety a dose of 0.09 mg/kg was

selected as it has previously been shown to enhance forward trace fear conditioning in C57BL/6J mice (Gould et al., 2004; Raybuck and Gould, 2009), and results in nicotine plasma levels similar to those found in smokers (Davis et al., 2005). Nicotine was administered intra-peritoneally, 2 min prior to training and testing. Administration prior to training and testing was based on prior work showing that acute systemic nicotine enhances trace fear conditioning when administered at training and testing (Davis and Gould, 2007).

For the chronic nicotine experiment, nicotine was delivered at 12.6 mg/kg per day for 14 days based on previous findings in mice that indicate that this dose of chronic nicotine lies within a range of doses (6.3–18 mg/kg per day) that result in plasma nicotine and cotinine levels similar to those of active smokers (Benowitz et al., 1988; Benowitz, 1996; Cole et al., 2014; Davis et al., 2005; Portugal et al., 2012b). In addition, this treatment schedule has previously been shown to model neural adaptations associated with chronic smoking, including upregulation of nicotinic receptors (Gould et al., 2012, 2014a).

Local administration—For local administration experiments, two doses of nicotine hydrogen tartrate were selected, 0.09 µg/ side and 0.18 µg/side (all doses reported in freebase). Nicotine hydrogen tartrate was dissolved in sterile saline, which was used as vehicle for control mice. Doses and administration parameters were based on prior work demonstrating that both are sufficient to enhance forward trace fear conditioning when infused into the dorsal hippocampus and prelimbic cortex prior to training and testing (Raybuck and Gould, 2010). Intracranial drug infusion procedures were similar to those previously described (Kenney et al., 2012b; Raybuck and Gould, 2010). Briefly, mice were restrained and dummy cannula removed. Drug was directly infused using 22 gauge internal cannula (dorsal hippocampus and ventral hippocampus) and 33 gauge internal cannula (prelimbic cortex) (Plastics One, Roanoke) attached to PE50 polyethylene tubing. Drug infusion rate for all three experiments was 0.5 µl/ min with a dosing volume of 0.5 µl per side. Infusions were controlled by microinfusion pump (KD Scientific, New Hope, PA, USA) with 10 µl Hamilton syringe (Reno, NV, USA). Internal cannulae remained in place for 1 min following infusion to allow for diffusion of drug. Mice were trained or tested immediately after infusion was complete.

Behavioral procedures

The dependent measure for all experiments, freezing, was defined as the absence of movement except for respiration (Blanchard and Blanchard, 1969). Freezing was measured using a time-sampling method, in which subjects were observed for 1 s every 10s. During each 1 s sample, mice were judged as freezing or active (Gould and Wehner, 1999). Freezing data were converted to and analyzed as % freezing. Finally, for all experiments, freezing was observed by experimenters blind to drug conditions.

For all studies, backward trace conditioning consisted of three training sessions occurring over the same number of days. The procedure was a CS+/CS– discrimination paradigm in which a signaled footshock is followed by an un-reinforced tone presentation (Figure 1). Within each session, five signaled US foot-shocks (2 s, 0.57 mA) were presented, followed by a 20 s trace interval. The footshock US was signaled by a CS+ (housetlight, 30 s) that co-

terminated with the US. Following the trace interval, a CS– (Tone, 6kHz, 85 dB) was presented. Each training session began with a 60 s baseline period prior to the first trial. The intertrial interval, range 90–120 s, was pseudorandomly assigned.

Behavioral testing for all experiments occurred in an alternate context 24 h after the last training session. Learned safety was assessed using summation testing in which learning of the CS as a conditioned inhibitor of fear was measured. Summation testing consisted of three alternating presentations of Light and Light/ Tone compound cues (60 s each), with 60 s intertrial intervals (Figure 1). Freezing was scored for the entire duration of cue presentation and data were collapsed into Light-Danger and Light/Tone-Safety for statistical analysis. Finally, in order to explicitly test whether nicotine caused the formation of a fear association with the backward CS, mice were tested for freezing to the backwards CS alone (Figure 1). Specifically, similar to Gould et al. (2004), mice were placed in an altered context and exposed to a 3 min stimulus free period, Pre-CS, followed by a 3 min presentation of the backward trace CS. Freezing was scored for the entire duration of testing during the first 3 min, Pre-CS, and the latter 3 min, CS, for statistical analysis.

Minipump surgeries

For the chronic nicotine study, mice were anesthetized with isoflurane (5% induction, 2.5% maintenance) and implanted with osmotic minipumps (Alzet, Model 1002, Durect Co, Cupertino, CA, USA). Minipumps delivered chronic saline or nicotine (12.6 mg/kg per day) for 14 days (testing occurring on the 14th day). Osmotic minipumps were surgically implanted subcutaneously via an incision posterior to the scapulae. The incision site was closed with surgical staples.

Cannula surgeries

After being anesthetized with isoflurane (5% induction, 2.5% maintenance), mice were placed in a stereotaxic apparatus and implanted with a guide cannula. The hippocampus is genetically and functionally differentiated along its septotemporal axis (Fanselow and Dong, 2010; Gould et al., 2014b). Therefore, bilateral guide cannulas (Plastics One) were placed for the dorsal hippocampus (A/P -1.7 , M/L ± 3.0 , D/V -2.3 mm) and the ventral hippocampus (A/P -2.8 , M/L ± 3.0 , D/V -4.0 mm) (Raybuck and Gould, 2010). The rodent prefrontal cortex is anatomically different from that of the primate, lacking granularization; however, the prelimbic cortex appears to mediate working memory and attention functions (Seamans et al., 2008; Uylings et al., 2003). In addition, the prelimbic cortex is critically involved in working memory-like activity during trace acquisition (Gilmartin et al., 2013b). Thus, prelimbic cortex was the target for PFC infusion. Coordinates were based on the stereotaxic location of prelimbic cortex (A/P $+1.7$, M/L ± 0.5 , D/V -2.5 mm) (Paxinos and Franklin, 2008). Mice were allowed to recover for at least five days prior to initiation of behavioral experimental procedures.

Histology

Following behavioral procedures mice were euthanized by cervical dislocation and brains were extracted. All brains were postfixed in formalin for a minimum of 24 h. Following fixation, brains were sectioned at 50 μ m on cryostat and stained with cresyl violet.

Confirmation of infusion sites was done using brightfield microscopy at 10× magnification to identify cannula tracks (Figures 2 and 3). Placements determined to fall outside of the target regions (23 mice total) were excluded from all analysis.

Data analysis

For all experiments freezing data were analyzed using mixed-design analyses of variance (ANOVAs) (SPSS 16.0). Exploratory analysis of prelimbic cortex infusion results also included one-way ANOVAs. Results were considered significant at $p < 0.05$. All data are presented as means \pm SEM. One animal from the backward trace fear conditioning experiment was removed from analysis due to freezing over two standard deviations from the mean. One animal from the chronic nicotine experiment and one from the dorsal hippocampus infusion experiment were removed for Pre-CS baseline freezing two standard deviations higher than the rest of the group.

Results

The effects of acute nicotine on backward trace conditioned safety

Mice were administered systemic nicotine on all sessions, training and testing, to assess changes in backward trace conditioned safety. A mixed-design ANOVA found a significant interaction (Drug (Nicotine vs. Saline) \times Condition (Light-Danger vs. Light/Tone-Safety)) $F_{(1,16)} = 7.910$, $p < 0.05$ and a main effect of drug, $F_{(1,16)} = 8.21$, $p < 0.05$. A planned comparison paired t -test indicated that saline treated control animals froze less during Light/Tone-Safety ($M = 29.01\%$, $SD = 14.64\%$) compared with Light-Danger ($M = 46.91\%$, $SD = 17.59\%$), $t(8) = 2.42$, $p < 0.05$. Thus, saline treated mice demonstrated conditioned inhibition of fear in response to the backward CS, indicating learned safety. In contrast, animals treated with nicotine showed no difference in freezing to Light-Danger ($M = 46.91\%$, $SD = 9.66\%$) versus Light/Tone-Safety trials ($M = 55.55\%$, $SD = 13.03\%$), $p > 0.05$. These data show that nicotine prevents responding to the backward CS as a conditioned inhibitor and disrupted backward trace conditioned safety. Finally, a planned independent-samples t -test found no difference between saline and nicotine treatment on freezing during baseline period, $p > 0.05$. Thus, both groups showed no differences in non-associative freezing behaviors (Figure 4).

The effects of chronic nicotine on backward trace conditioned safety

To assess whether chronic nicotine might also disrupt learned safety we performed the same backward trace conditioned safety procedure on mice chronically administered nicotine via osmotic mini-pumps. Mixed-design ANOVA showed a within subjects main effect of testing condition (Light-Danger vs. Light/Tone-Safety) $F_{(1,27)} = 28.52$, $p < 0.01$, but no significant between subjects main effect of drug (Nicotine vs. Saline) $F_{(1,28)} = 0.46$, $p > 0.05$ or interaction was observed (Condition \times Drug) $F_{(1,27)} = 0.53$, $p > 0.05$. The significant between subjects main effect indicates that both saline and nicotine treatment groups learned safety. Specifically, chronic saline treated mice froze more during the Light-Danger ($M = 47.62\%$, $SD = 12.65\%$) compared with Light/Tone-Safety ($M = 30.55\%$, $SD = 14.24\%$). Similarly, chronic nicotine treated mice also showed freezing at lower levels during Light/Tone-Safety ($M = 29.63\%$, $SD = 15.24\%$) compared with the Light-Danger ($M = 42.59\%$,

SD = 13.87%) (Figure 4). Finally, an independent samples *t*-test found that nicotine treated mice froze significantly more during the Pre-CS period compared with saline treated mice ($t(27) = 2.93, p < 0.05$). However, because nicotine and saline treated mice both showed similar high levels of freezing during Light-Danger, indicating formation of a fear association with the forward CS, differences in Pre-CS freezing did not alter our interpretation of the mixed ANOVA results, which suggest that both groups learned the safety association.

The effects of nicotine on backward trace fear conditioning

In order to assess whether acute nicotine disrupts backward trace conditioned safety by causing a fear association with the backward conditioned cue an alternative test was implemented. Rather than a summation test, mice were placed in an alternative context and presented with the backward conditioned cue alone. Thus, if mice learned the cue as safe they should show no freezing, while freezing to the cue would indicate a fear association. A mixed-design ANOVA found a significant effect of condition (Pre-CS vs. CS) $F_{(1,13)} = 5.82, p < 0.05$. In addition, a trending but non-significant interaction was found, $F_{(1,13)} = 4.19, p = 0.061$, while no significant between subjects effect of drug was observed, $p > 0.05$. Importantly, as hypothesized, a planned comparison paired-samples *t*-test found that nicotine treated mice froze significantly more during presentation of CS ($M = 29.16\%$, $SD = 14.47\%$) than during the Pre-CS period ($M = 9.72\%$, $SD = 14.16\%$) ($t(7) = 2.65, p < 0.05$) (Figure 5). Therefore, nicotine treated mice formed a fear association with the backward conditioned cue. In contrast, there was no difference observed in the saline group: Pre-CS ($M = 8.73\%$, $SD = 12.36\%$) and CS ($M = 10.31\%$, $SD = 8.74\%$), $p > 0.05$. These data support the claim that acute nicotine can disrupt learned safety by facilitating maladaptive associations between the CS and the footshock US.

The effects of local administration of nicotine into the dorsal hippocampus on backward trace conditioning

Similar to the previously described systemic experiments, backward trace conditioned safety was assessed using a summation test. Nicotine (0.09 and 0.18 $\mu\text{g}/\text{side}$) or saline was infused into the dorsal hippocampus prior to training and testing. A mixed-design ANOVA found a significant interaction (Drug \times Condition), $F_{(2,20)} = 3.79, p < 0.05$. Also a within subjects main effect of testing condition was found (Danger vs. Safe), $F_{(1,20)} = 14.46, p < 0.01$, but no between subjects main effect was observed (Saline vs. 0.09 μg vs. 0.18 μg), $F_{(2,20)} = 3.145, p = 0.07$. A priori planned contrasts using paired samples *t*-test found that mice administered saline showed significant decreased freezing during Light/Tone (27.16%, $SD = 14.28\%$) compared with Light alone (42.59%, $SD = 10.39\%$), $t(8) = 3.22, p < 0.05$. Similarly, mice infused with 0.18 $\mu\text{g}/\text{side}$ nicotine also froze significantly less during Light/Tone (29.37%, $SD = 8.91\%$) compared with Light alone (41.27%, $SD = 14.99\%$), $t(6) = 3.19, p < 0.05$. In contrast, mice administered 0.09 $\mu\text{g}/\text{side}$ nicotine did not freeze differently during Light/Tone (50.00%, $SD = 20.54\%$) compared with Light alone (50.00%, $SD = 15.04\%$), $p > 0.05$. Finally, a one-way ANOVA revealed no significant variation in Pre-CS freezing among the different drug groups, $F_{(2,22)} = 0.237, p > 0.05$. Thus, no group differed in innate freezing or generalized freezing to the testing context.

The effects of local administration of nicotine into the ventral hippocampus on backward trace conditioning

To examine whether the ventral hippocampus plays a role in the effects of acute nicotine on backward trace conditioning, nicotine (0.09 and 0.18 $\mu\text{g}/\text{side}$) or saline was infused into the ventral hippocampus prior to training and testing. A mixed-design ANOVA found a significant within subjects main effect of testing condition (Danger vs. Safety), $F_{(1, 20)} = 43.02$, $p < 0.01$, indicating that mice responded differently to Light-Danger and Light/Tone-Safety presentations. No between subjects main effect of drug (Saline vs. 0.09 μg vs. 0.18 μg), $F_{(2, 20)} = 2.665$, $p > 0.05$ or interaction was observed, $F_{(2, 20)} = 0.036$, $p > 0.05$. The significant within subjects main effect of testing condition indicates that all saline and nicotine treatment groups learned safety. Specifically, mice administered saline froze less during compound Light/ Tone-Safety (24.07%, SD = 16.43%) compared with Light-Danger (40.12%, SD = 13.26%). This effect of testing condition was also observed in mice infused with 0.09 μg nicotine, Light/ Tone-Safety (14.29%, SD = 8.99%) compared with Light-Danger (31.75%, SD = 9.99%), and 0.18 μg nicotine, Light/Tone-Safety (26.19%, SD = 7.67%) compared with Light-Danger (43.65%, SD = 10.36%). Finally, assessment of Pre-CS freezing using a one-way ANOVA was not significant, $F_{(2,22)} = 0.883$, $p > 0.05$. Therefore, groups did not differ in innate freezing or show differences in generalized freezing from the testing context (Figure 6).

The effects of local administration of nicotine into prelimbic cortex on backwards trace conditioning

To assess whether the prelimbic cortex is sufficient to mediate the effects of acute nicotine on backward trace conditioning, nicotine (0.09 and 0.18 $\mu\text{g}/\text{side}$) or saline was directly infused into the prelimbic cortex prior to training and testing. A mixed-design ANOVA revealed a significant interaction (Condition \times Drug), $F_{(2,22)} = 4.48$, $p < 0.05$. In addition, a main effect of testing condition was found (Danger vs. Safety), $F_{(1,22)} = 130.29$, $p < 0.01$, but there was no significant between subjects main effect of drug (Saline vs. 0.09 μg vs. 0.18 μg), $F_{(2,22)} = 2.68$, $p > 0.05$. Therefore, the effect cue presentation on freezing was moderated by nicotine treatment. A priori planned paired samples t -tests were performed and saline treatment was associated with significantly reduced freezing during Light/Tone-Safety trials (13.89%, SD = 8.91%) compared with Light-Danger (45.14%, SD = 13.09%), $t(7) = 9.00$, $p < 0.01$. Additionally, mice treated with 0.09 $\mu\text{g}/\text{side}$ nicotine showed significantly reduced freezing during Light/Tone-Safety (37.04%, SD = 19.64%) compared with Light-Danger (53.09%, SD = 17.37%), $t(8) = 4.73$, $p < 0.01$. Similarly, mice treated with 0.18 $\mu\text{g}/\text{side}$ nicotine showed significantly reduced freezing during Light/Tone-Safety (26.38%, SD = 14.47%) compared with Light-Danger (52.77%, SD = 12.59%), $t(7) = 6.14$, $p < 0.01$ (Figure 6).

Our a priori comparisons found that all drug groups inhibited fear during Light/Tone-Safety compared with Light-Danger. However, visual inspection of the data, as well as a significant interaction from the mixed ANOVA, suggested that drug groups froze differently during presentation of compound Light/Tone. To further examine this, we performed a one-way ANOVA on freezing data from Light/Tone-Safety and found a significant effect of drug, $F_{(2,25)} = 4.88$, $p < 0.05$. Furthermore, follow up post-hoc contrasts using Tukey's HSD

(honest significant difference) showed that treatment with nicotine 0.09 µg/side resulted in significantly higher freezing compared with saline treatment, $p < 0.05$, during Light/Tone trials. No significant difference was found between saline and 0.18 µg/side nicotine, $p > 0.05$. We considered that this difference could be the result of a global reduction in freezing within the control group. To assess this, we also performed a one-way ANOVA for Light-Danger and found a non-significant effect of drug, $F_{(2,25)} = 0.77$, $p > 0.05$. Finally, a one-way ANOVA found no significant differences in freezing during the Pre-CS period, $F_{(2,24)} = 1.48$, $p > 0.05$, indicating that mice did not differ in innate freezing. These data suggest a modest effect of nicotine within the prelimbic cortex on inhibitory summation of fear.

Discussion

The present study examined the effects of nicotine on backward trace conditioned safety and found that acute nicotine disrupted safety learning, observed as a failure to reduce freezing to a danger CS+ in the presence of the backward CS- during summation testing. Next, we assessed whether acute nicotine-dependent disruption of learned safety was the result of a US-CS danger association. We found that mice treated with acute nicotine displayed a fear response to the backward CS in a novel context. Thus, acute nicotine prevented learning of the backward CS as a safety cue by facilitating the formation of a maladaptive fear association with a backward CS.

Within the context of prior work investigating the effects of acute nicotine on learning, the finding that acute nicotine disrupts safety learning initially appears divergent. For example, acute nicotine has been repeatedly shown to enhance hippocampus-dependent forms of learning (Davis et al., 2005; Gould and Wehner, 1999; Kenney et al., 2011). However, our findings suggest that this disruption is actually due to nicotine facilitating the formation of a maladaptive US-CS association; that is, the backward trace conditioning. Normally, backward trace conditioning either does not produce learning or produces weak associations (Quinn et al., 2002). Nicotine enhances hippocampus-dependent learning, including trace fear conditioning (Gould et al., 2004; Raybuck and Gould, 2009), and backward trace conditioning may engage the hippocampus (Quinn et al., 2002). In support, a previous study found that post-training lesions of the dorsal hippocampus abolished recall of forward as well as backward trace fear associations (Quinn et al., 2002), which suggests that dorsal hippocampus-dependent processes may similarly mediate forward trace fear conditioning as well as backward trace conditioned fear associations. Thus, acute nicotine may facilitate backward trace fear associations via hippocampal learning processes that become engaged when stimuli are discontinuous, which results in the disruption of a competing safety association.

In contrast to the effects of acute nicotine, we observed no effect of chronic nicotine on backward trace conditioned safety. This null finding supports prior studies showing that while initial nicotine exposure leads to changes in learning, chronic administration results in tolerance to such cognitive effects (Davis et al., 2005; Portugal et al., 2012a). Tolerance is thought to be mediated by neural adaptations induced by continuous nicotine exposure, including desensitization and upregulation of nAChRs (Marks et al., 1992, 1993). Behavioral work supports this view as the rate at which tolerance develops has been shown

to temporally align with nAChR upregulation within the hippocampus (Gould et al., 2014a). Furthermore, acute nicotine has been shown to facilitate trace conditioning, but chronic nicotine does not (Raybuck and Gould, 2009). Therefore, the lack of an effect of chronic nicotine on backward trace conditioned safety is consistent with our interpretation that acute nicotine facilitates a maladaptive hippocampus-dependent trace US–CS association. A caveat is that only a single dose of nicotine was tested, but this dose falls within a range of doses resulting in similar plasma nicotine and cotinine levels as observed in active human smokers (Cole et al., 2014; Hukkanen et al., 2005; Kutlu et al., 2016b).

In follow up to experiments that revealed an effect of acute nicotine on backward trace conditioning, we found that nicotine infused into the dorsal hippocampus, a region involved in trace conditioning, also resulted in similar disruption of backward trace conditioned safety learning. Specifically, we found that infusion of nicotine at 0.09 µg/side into the dorsal hippocampus disrupted inhibitory summation. These data suggest that nicotine may disrupt safety via changes in dorsal hippocampus process underlying associative learning. In support, post-training lesions of the dorsal hippocampus abolished backward trace conditioned fear (Quinn et al., 2002). In addition, local administration of nicotine within the dorsal hippocampus during training enhanced trace fear conditioning (Raybuck and Gould, 2010). Thus, nicotine may act to enhance the US–CS association by altering signaling within the dorsal hippocampus that mediates learning temporally discontinuous trace memories. Therefore, our results give evidence that nicotine can alter associative learning by acting directly within the dorsal hippocampus and that this can alter adaptive safety learning by facilitating maladaptive associations.

In contrast to dorsal hippocampus infusions, ventral hippocampus administration had no effect on backward conditioned safety. This null effect supports the interpretation that nicotine's effects on backward trace conditioning are mediated by enhanced maladaptive associative learning. For example, previous studies have found that infusion of nicotine into the ventral hippocampus actually disrupts associative learning (Kenney et al., 2012b; Raybuck and Gould, 2010). Therefore infusion of nicotine into the ventral hippocampus likely has no effect on safety because under normal conditions backward trace conditioning results in no excitatory US–CS association. Our results suggest that the ventral hippocampus may not be a critical mediator of learned safety. This conclusion is also directly supported by work showing that inactivation of the ventral hippocampus does not prevent discrimination of dangerous and safe cues (Chen et al., 2016). Thus, while the ventral hippocampus plays an important role in fear expression, as observed in other fear conditioning and fear extinction work (Kjelstrup et al., 2002; Sierra-Mercado et al., 2011), our data support a view that it may not similarly modulate learned safety. In sum, these data suggest that the ventral hippocampus is not important in the effect of systemic nicotine on backward conditioned safety.

Given that the prelimbic cortex may play a role in cue discrimination as well as trace conditioning (Connor and Gould, 2016; Gilmartin et al., 2013a, 2013b; Likhtik et al., 2014; Meyer and Bucci, 2014), we infused nicotine into the prelimbic cortex to assess nicotine's effect on backward trace conditioned safety. We found that all groups showed inhibitory summation, indicating nicotine did not prevent learned safety. However, we also observed a

small increase in freezing during summation testing in mice receiving the lower dose, 0.09 µl/side. This modest effect of nicotine within the prelimbic cortex appears consistent with other investigations of conditioned safety, which indicate that lesions or inactivation of the mPFC result in null (Christianson et al., 2008; Gewirtz et al., 1997) or small effects (Sangha et al., 2014). Alternatively, cells within the prelimbic cortex maintain sustained firing during the trace interval during forward trace fear conditioning, suggesting that the prelimbic cortex mediates learning non-overlapping events by maintaining a representation of separate events across time (Gilmartin and McEchron, 2005) and inhibition of prelimbic cortex neurons during the trace interval disrupted trace fear learning (Gilmartin et al., 2013b). Moreover, nicotine can modulate theta and mPFC-amygdala theta connectivity is associated with danger/safe cue discrimination (Bueno-Junior et al., 2012; Likhtik et al., 2014). Similarly, nicotine can enhance working memory, which involves short-term maintenance of information (Levin and Torry, 1996; Provost and Woodward, 1991). Therefore, during backward trace conditioning, nicotine may have altered prelimbic cortex activity during the trace interval increasing the salience of the US across the trace interval and decreasing the salience of the safety cue.

Our results from the direct infusions studies demonstrating efficacy at the lower dose are in agreement with previous behavioral work showing that the effects of nicotine are associated with an inverse U-shaped dose–response curve (Picciotto, 2003). For example, systemic treatment with acute nicotine results in enhanced contextual fear conditioning in a dose-dependent inverted-U shaped fashion (Gould and Higgins, 2003). Similarly, enhancement of forward trace fear conditioning by dorsal hippocampus and mPFC nicotine infusions also showed an inverted U-shaped dose–response (Raybuck and Gould, 2010). One possible reason for a U-shaped dose–response to nicotine is that nAChRs may activate both inhibitory and excitatory processes (Picciotto, 2003), mediated by different nAChR subtypes that have differential affinity for nicotine. Indeed, lower levels of nicotine, similar to that seen in smokers, are thought to act via high-affinity nAChRs (Wooltorton et al., 2003). Therefore, a higher dose of nicotine might additionally activate low-affinity nAChRs, changing the pharmacological effects of nicotine.

This present study was not designed to determine whether the effects of nicotine on safety learning depend on changes of acquisition or recall. However, prior work has shown that the enhancing effects of nicotine on fear learning are dependent on molecular substrates of long-term memory consolidation (Kenney et al., 2012a), and blockade of protein synthesis prevents long-term enhancement of contextual fear memory by systemic nicotine administration (Gould et al., 2014b). Additionally, administration of nicotine prior to testing did not enhance hippocampal fear memory recall (Gould and Wehner, 1999). Similarly, direct infusion of nicotine into the mPFC prior to testing did not alter freezing after trace fear conditioning (Raybuck and Gould, 2010) and pre-testing administration of nicotine into dorsal hippocampus did not alter freezing after contextual fear conditioning (Kenney et al., 2012b). Thus, the effects of nicotine on learned safety could be due to changes in memory acquisition/consolidation; however, future studies are needed to explicitly examine this.

This study shows for the first time that nicotine can alter cued safety learning, a process that appears highly adaptive, but dysfunctional in individuals with PTSD. Thus, these results

may have implication for understanding the relationship between nicotine use and PTSD, particularly with regard to nicotine exposure during smoking initiation in naïve individuals. In support, clinical work suggests that the rates of smoking initiation are higher in individuals with PTSD (Fu et al., 2007). Other preclinical studies have found that acute nicotine can also disrupt fear extinction and contextual safety discrimination (Gould and Kutlu, 2014; Kutlu et al., 2014). Together these data indicate that smoking initiation during or after a traumatic event may facilitate or exacerbate PTSD symptomology. In sum, while there are likely multiple ways in which nicotine use may alter cognition to facilitate PTSD symptomology, we show that under some conditions nicotine can alter learning of cues that indicate safety by facilitating formation of maladaptive danger associations with cues that actually predict the absence of threat.

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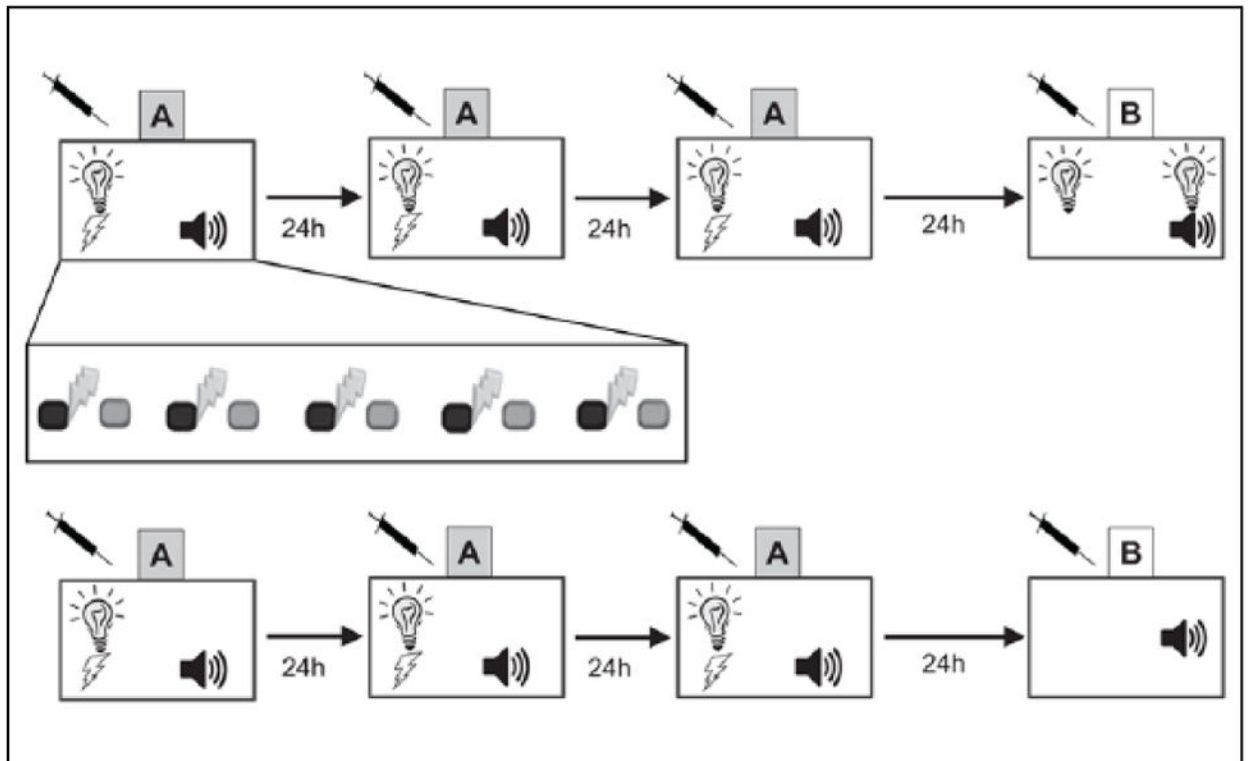


Figure 1.

Design schematics for training and testing of backwards trace conditioned safety and fear.

(a) Mice were trained over three days in context A with five trials. Exploded view shows temporal arrangement of cues during training, with a 30 s light co-terminating with 2 s footshock (dark gray with thunderbolt) followed by a 20 s trace interval and a 30 s tone (light gray). To test for learned safety, summation testing consisted of alternating presentations of light or light/tone. (b) To assess backwards trace conditioned fear mice were trained similarly, but on testing were only presented with the backward paired tone conditioned stimulus.

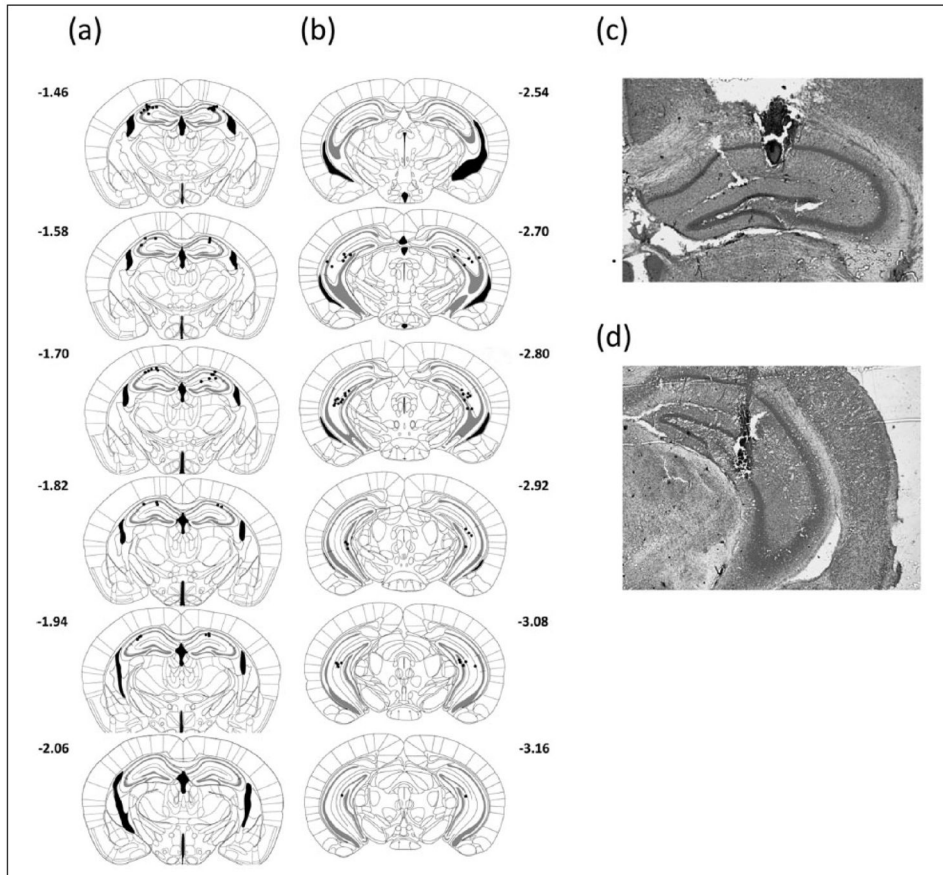


Figure 2. Cannula placement in hippocampus. Cannula tips location in relation to bregma in coronal sections (a) dorsal and (b) ventral hippocampus. Representative images of placements in dorsal (c) and ventral (d) hippocampus.

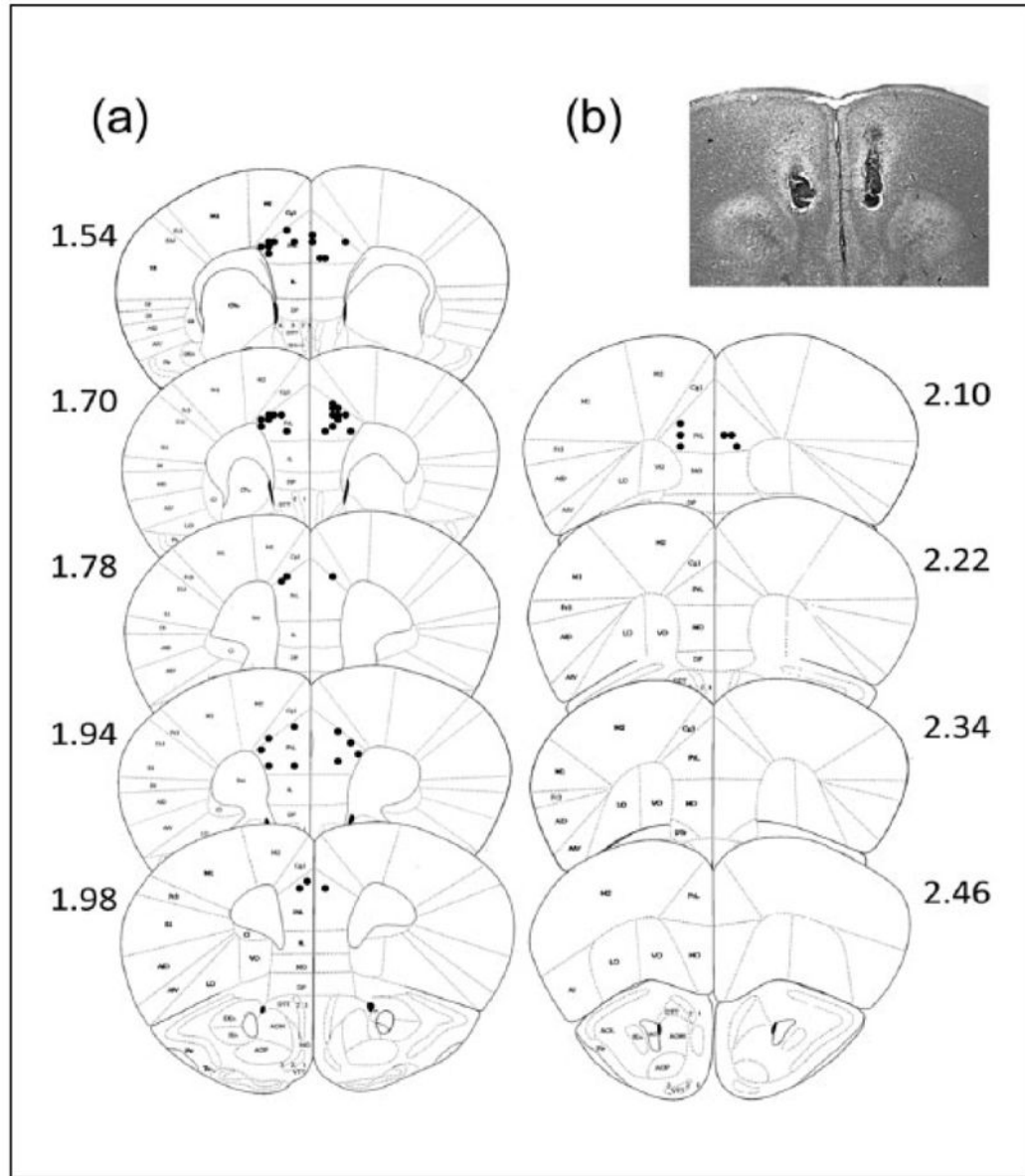


Figure 3. (a) Cannula placement in prelimbic cortex with (b) representative image. Cannula tips location in relation to bregma in coronal sections.

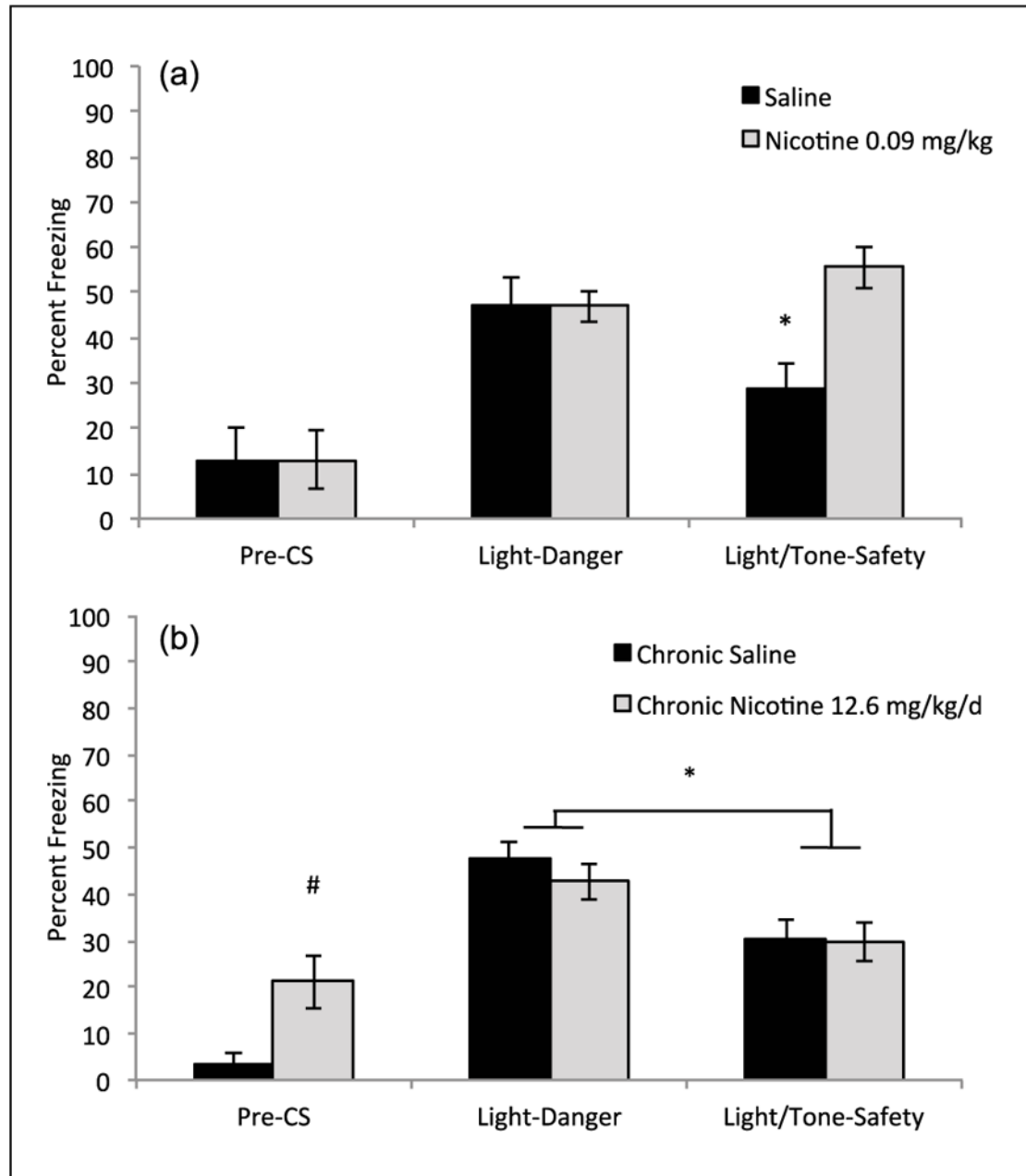


Figure 4.

The effects of acute and chronic nicotine on backward trace conditioned safety. (a) Mice treated with acute nicotine, 0.09 mg/kg, did not freeze less during summation testing of the Light/Tone-Safety compound cue, but saline treated mice froze significantly less (data shown as % freezing in mice) during compound Light/Tone compared with Light alone ($n = 9$ per group). Error bars indicate SEM, * indicates within drug group difference between Light-Danger and Light/Tone-Safety, $p < 0.05$. (b) Mice in both chronic saline and chronic nicotine, 12.6 mg/kg/ per day, demonstrated reduced freezing during presentation of Light/Tone-Safety compared with Light alone ($n = 14$ per group). Mice treated with chronic

nicotine showed greater generalized freezing during the Pre-CS period prior to stimulus presentation. Error bars indicate SEM, * indicates a main effect of testing condition Light-Danger versus Light/ Tone-Safety, $p < 0.05$. # indicates significant difference between chronic saline and nicotine during Pre-CS, $p < 0.05$.

CS: conditioned stimulus

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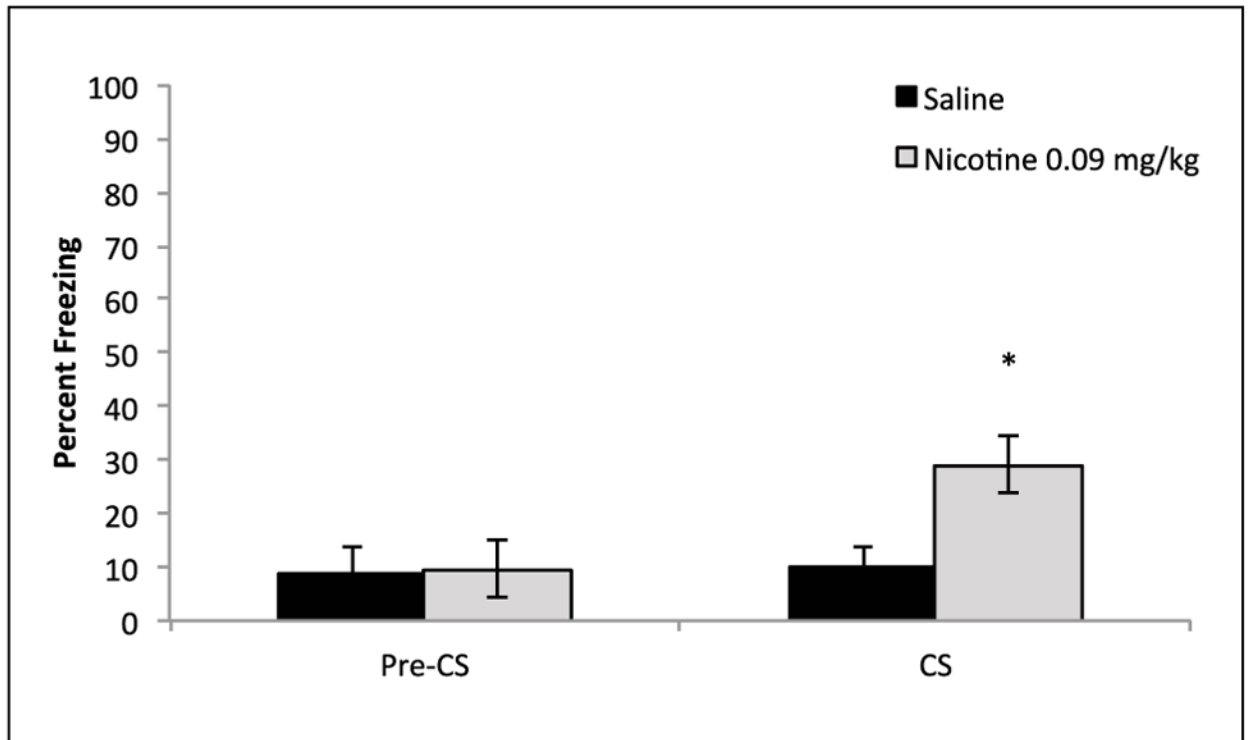


Figure 5.

Backward trace fear conditioning control mice did not show any difference in freezing during the Pre-CS versus CS conditioning, indicating no fear association with the backward trace conditioned CS. In contrast, acute nicotine, 0.09 mg/kg, treatment resulted in greater freezing to the CS compared with Pre-CS, indicating a fear association with the backward trace conditioned CS ($n = 7-8$). Error bars indicate SEM, * indicates significant planned comparison paired samples t -test between Pre-CS and CS, $p < 0.05$.

CS: conditioned stimulus

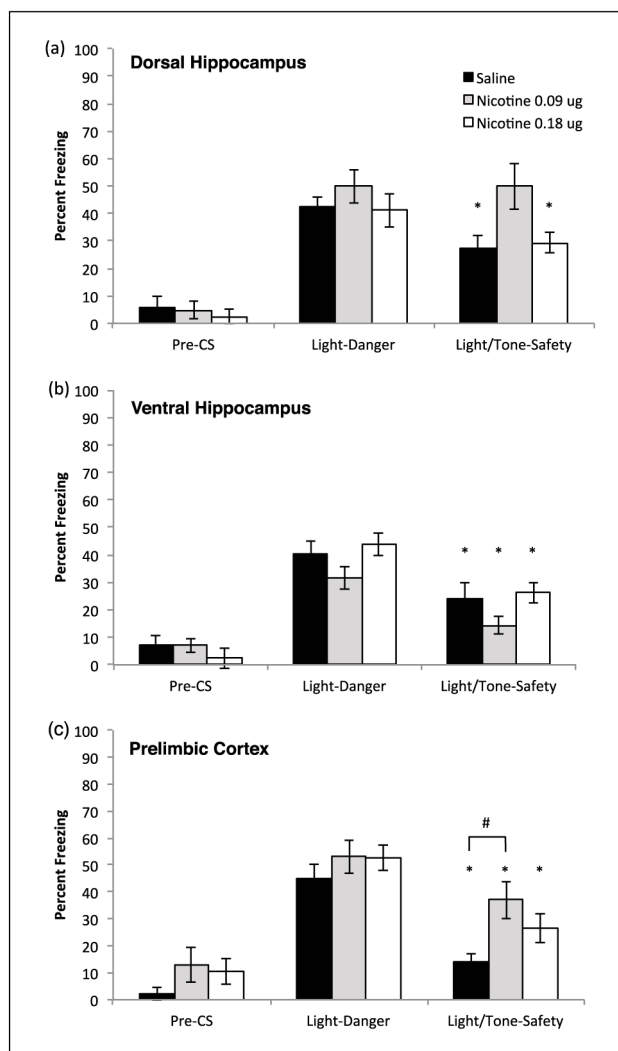


Figure 6.

(a) Nicotine, in the dorsal hippocampus dose-dependently disrupts safety learning. Mice treated with 0.09 $\mu\text{g}/\text{side}$ nicotine fail to show learned safety, with freezing levels similar between Light-Danger and Light/Tone-Safety. Mice treated with saline and nicotine, 0.18 $\mu\text{g}/\text{side}$, showed learned safety with decreased freezing during Light/Tone-Safety ($n = 9-7$ per group). Error bars indicate SEM, * indicates significant within group planned comparison paired samples t -test between Light-Danger and Light/Tone-Safety, $p < 0.05$. (b) Nicotine in the ventral hippocampus has no effect on backwards trace conditioned safety; all mice showed decreased freezing during Light/ Tone-Safety compared with Light-Danger ($n = 9-7$ per group). Error bars indicate SEM, * indicates significant within group difference between Light and Light/Tone, $p < 0.05$. (c) Nicotine in prelimbic cortex dose-dependently alters freezing to Light/Tone compound. Mice administered saline 0.09 $\mu\text{g}/\text{side}$ and nicotine 0.18 $\mu\text{g}/\text{side}$ all showed significantly less freezing during presentation of compound Light/ Tone-Safety compared with Light-Danger. However, mice treated with 0.09 $\mu\text{g}/\text{side}$ nicotine froze significantly more than saline treated mice during compound Light/Tone-Safety

presentation ($n = 8-9$ per group). Error bars indicate SEM, * indicates planned comparison paired samples t -test $p < 0.05$, # indicates post-hoc Tukey's HSD contrast $p < 0.05$. CS: conditioned stimulus; HSD: honest significant difference

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