ACUTE NICOTINE-DEPENDENT ALTERATIONS IN ASSOCIATIVE LEARNING INTERFERE WITH BACKWARDS TRACE CONDITIONED SAFETY

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ABSTRACT

Acute Nicotine-dependent Alterations in Associative Learning Interfere with Backwards Trace Conditioned Safety

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Organisms can form safety associations with cues that predict the absence of an aversive event. This cognitive process, learned safety, is important for modulating emotional processing, as safety cues can decrease fear in the presence of previously learned danger cues. Further, there are clinical implications in understanding learned safety, as individuals with PTSD present with deficits in learned safety. Additionally, there is a well established relationship between smoking and PTSD. The link between smoking and PTSD is unclear, however one possibility is that nicotine-associated changes in cognition could facilitate PTSD symptoms, particularly by disrupting are altering learned safety. Considering that nicotine has been shown to modulate associative learning, including hippocampus-dependent forms of fear learning, we hypothesized that nicotine administration could cause maladaptive associative learning to occur, leading to altered safety learning. In the present study, mice were administered acute nicotine and trained and tested in two forms of cued safety learning, explicitly unpaired and backwards trace conditioning. To test for conditioned inhibition of fear by safety cues we performed summation testing. Summation testing indicated that acute nicotine did

not impact unpaired learned safety, but did disrupt backwards trace conditioned safety. Additionally, chronic nicotine was found to have no effect on backwards trace conditioned safety, suggesting the development of tolerance. Importantly, on a separate test in which the backwards trace conditioned stimulus was presented alone in a novel context, acute nicotine administration was found to facilitate a fear association with the backwards trace conditioned stimulus. Therefore, acute nicotine prevented backwards trace conditioned safety, by facilitating the formation of a maladaptive fear association. Finally, we found that infusion of nicotine into the dorsal hippocampus and medial prefrontal cortex resulted in similar maladaptive behavioral patterns in summation testing. These findings are discussed with respect to how nicotine can alter cognition and the role alterations in cognition may play PTSD.

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TABLE OF CONTENTS

LIST OF FIGURES

Figure Page

CHAPTER 1

INTRODUCTION

The ability to modulate and inhibit emotional learning processes is critical for normal functioning. Associative learning is recruited to mediate adaptive modulation of emotional processes. For example, learning environmental features that correctly predict danger is fundamental for adaptive behavioral responding (McNally & Westbrook, 2006). As a result, Pavlovian fear learning in which a conditioned stimulus (CS) is associated with an aversive unconditioned stimulus (US), has been widely studied (Phillips & LeDoux, 1994). Importantly, associative learning can also result in the formation of safety associations with cues that predict the absence of aversive stimuli. This type of learning, learned safety, constitutes a form of conditioned inhibition (Christianson et al., 2012; Rescorla, 1969). Thus, as a conditioned inhibitor of fear, learned safety cues can decrease fear responding in the presence of danger cues. Similar to fear learning, which has been demonstrated in rodents and humans, learned safety appears well conserved, and has been observed drosophila, rodents, and humans (Christianson et al., 2011; Jovanovic, Kazama, Bachevalier, & Davis, 2012; Yarali, Nehrkorn, Tanimoto, & Herz, 2012).

Disruptions in an individual's ability to modulate fear-related emotional processes may underlie stress and anxiety-related disorders such as post-traumatic stress disorder (PTSD) (VanElzakker, Kathryn Dahlgren, Caroline Davis, Dubois, & Shin, 2014). For example, PTSD symptomology has been shown to include failure to

inhibit fear to cues indicating the absence of danger (Jovanovic et al., 2009). Moreover, failure to inhibit fear is thought to be related to disruptions in learned safety (Jovanovic, Norrholm, Blanding, Davis, et al., 2010; Jovanovic et al., 2012). More specifically, individuals with PTSD showed deficits in an AX+/BX- discrimination paradigm (Jovanovic et al., 2009; Jovanovic, Norrholm, Blanding, Davis, et al., 2010). In this paradigm, using fear potentiated startle, Jovanovic and colleagues found that individuals with PTSD had a similar response to a cue indicating the absence of aversive stimulus, B trials, and to a cue that indicated danger, A trials. Thus, participants with PTSD were unable to discriminate between danger and safety cues. Additionally, Jovanovic and colleagues also found that individuals with PTSD failed to show reduced fear to a danger cue when a safety cue was co-presented, indicating that these individuals did not form an inhibitory association with the safety cue (Jovanovic et al., 2012). Taken together, these data suggest a relationship between the ability to learn and respond to safety cues and PTSD. Resultantly, failure to learn safety has been proposed as a behavioral biomarker for PTSD (Jovanovic, Norrholm, Blanding, Davis, et al., 2010). It is currently unclear if disruptions in learned safety predisposes an individual to PTSD or if disruptions in safety co-occur with development of PTSD symptoms. However, work suggests that increased levels of anxiety may lead to deficits in learned safety (Liao & Craske, 2013), and levels of fear-potentiated startle in response to a safety cue positively predicted anxiety levels (Jovanovic et al., 2014). Therefore, the relationship may be bidirectional; with PTSD-associated disruptions in learned

safety reflecting dysfunctional changes in emotional processing and changes in learned safety negatively impacting emotional processing.

PTSD is a psychological disorder that develops after exposure to a traumatic event(s) and is characterized by re-experiencing trauma, avoidance of trauma associated cues, and increased arousal (Jovanovic, Norrholm, Blanding, Davis, et al., 2010; Rothbaum & Davis, 2003). An estimated 9.2 percent of individuals exposed to a traumatic experience may develop PTSD (Breslau et al., 1998). Much work investigating the impact of PTSD on the individual has focused on war veterans. Such data suggest that trauma exposed veterans are at increased risk of suicide and substance abuse (Bremner, Southwick, Darnell, & Charney, 1996; Hendin & Haas, 1991). However, the risks of PTSD are not limited to veterans and are found to be highly problematic in civilian populations as well. For example, civilian individuals with PTSD are at a greater risk of attempting suicide (9.6%) compared to the general population (1.1 - 4.6%) (Kessler, Borges, & Walters, 1999; Tarrier & Gregg, 2004). Such findings support the need to better understand factors that contribute to the development and maintenance of PTSD. While there are likely many factors involved with the development and maintenance of PTSD symptoms, evidence suggests that PTSD is associated with deficits in learning and memory processes (Elzinga & Bremner, 2002). In particular, PTSD has been associated with changes in hippocampus-dependent declarative memory (Acheson, Gresack, & Risbrough, 2012). Further, human imaging studies suggest PTSD is associated with learning-dependent disruptions of PFC and hippocampal function (Astur et al., 2006; Bryant et al., 2008; Liberzon & Sripada, 2007; Rougemont-Bücking et al., 2011), regions

also implicated in learned safety (Kong, Monje, Hirsch, & Pollak, 2014). Therefore, understanding the development and maintenance of PTSD symptomology will likely require investigation changes in cognition that modulate emotion, such as learned safety.

While learned safety appears to be a unique cognitive process, it is likely mediated in part by mechanisms involved in emotional learning more generally. In particular, the neural mechanisms of fear learning likely overlap with those of learned safety. In support, fear learning processes are intrinsic to learned safety, insomuch as a cue becomes a salient indicator of safety in relation to the occurrence of an aversive event. Thus, fear learning processes are inherently engaged during learned safety. In addition, work has shown that safety learning leads to reduce neural activity, measured by c-Fos, within the amygdala, a brain region critical for fear learning and fear expression (Christianson et al., 2011; Phillips & LeDoux, 1992). Such data suggests that learned safety acts in opposition to fear learning processes within the amygdala. Learned safety may also recruit hippocampal processes, a brain region critical for some forms of fear learning and fear inhibition (Corcoran & Maren, 2001). For example, learned safety was found to result in increased hippocampal cell survival and BDNF levels, both of which are important for hippocampus-dependent associative learning (Liu, Lyons, Mamounas, & Thompson, 2004; Pollak et al., 2008; Shors et al., 2001). Anatomically, the hippocampus has direct connections to the amygdala (Pitkänen, Pikkarainen, Nurminen, & Ylinen, 2000) and hippocampal projections can elicit changes in plasticity within the basolateral amygdala (Maren & Fanselow, 1995). It

is thought that such hippocampal-amygdala connections are necessary for hippocampus-dependent contextual fear learning (Fanselow, 2010). Thus, altered hippocampus-dependent learning may influence changes in emotion-evoked amygdala-dependent processes and could possibly alter safety learning via changes in hippocampal-amygdala circuits.

Considering that learning of fear and safety may depend on overlapping associative mnemonic processes and neural substrates, drugs that alter associative learning processes might disrupt or alter safety learning. One such substance is nicotine, the psychoactive component found in tobacco products (Markou, 2008). Indeed, much work has revealed that nicotine can alter cognition, in both clinical populations and preclinical models (Amitai & Markou, 2009; Gould et al., 2012; Kenney, Raybuck, & Gould, 2012; Levin et al., 1990; Rezvani & Levin, 2001). Moreover, numerous studies also suggest a robust association between smoking and stress/anxiety disorders, including PTSD (see for review, Cougle et al., 2010; Feldner, Babson, & Zvolensky, 2007). Therefore, a possible causal link in the relationship between PTSD and smoking may be that nicotine-associated changes in cognition lead to deficits in learned safety.

CHAPTER 2

THE EFFECTS OF SYSTEMIC NICOTINE ON LEARNED SAFETY

Rationale

Deficits in learned safety are associated with PTSD, therefore understanding cognitive effects of nicotine on learned safety may be important in understanding the relationship between tobacco use and PTSD. Specifically, acute nicotine exposure in nicotine-naïve populations may be problematic. For example, smoking initiation is greatly increased amongst military personnel deployed in combat, at 1.6 times that of a normal non-smoker (Smith et al., 2008). Considering that combat exposure alone constitutes a significant risk factor for development of PTSD (Lapierre, 2008), co-occurrence of smoking initiation and trauma might lead to exacerbation of PTSD symptoms. Additionally, epidemiological evidence indicates that chronic smoking post-trauma is a risk factor for PTSD (Velden, Grievink, Olff, Gersns, & Kleber, 2007). Therefore, chronic nicotine exposure might also alter learned safety and facilitate PTSD symptomology.

In preclinical models, acute nicotine administration has been shown to alter hippocampus-dependent associative learning, leading to enhanced contextual and trace fear conditioning (Gould & Wehner, 1999; Raybuck & Gould, 2009). Here we sought to understand if acute nicotine alters learned safety in two ways. (1) Acute nicotine-associated enhanced learning of contextual cues indicating danger would interfere with learning a discrete safety cue within the same context. (2) Acute nicotine altered learning of temporally discontiguous stimuli would lead to a

change in associative strength of a safety cue. Investigating the ability of nicotine to disrupt learned safety is supported by prior findings indicating that acute nicotine disrupts inhibition of fear. Specifically, acute nicotine administration has been shown to disrupt discrimination of safe vs. dangerous contexts in which an animal distinguishes between a context paired with an aversive stimulus and an unpaired context (Kutlu, Oliver, & Gould, 2014). Additionally, acute nicotine has been shown to disrupt contextual fear extinction (Kutlu & Gould, 2014). Taken together, these findings suggest that acute nicotine may alter hippocampal learning, facilitating maladaptive danger associations or disrupting formation of adaptive safety associations.

In contrast to the effects of acute nicotine on hippocampus-dependent fear learning, chronic nicotine has been shown to result in development of tolerance. For example, mice treated with chronic nicotine for 12 days via osmotic mini-pumps showed normal learning of contextual and trace fear conditioning (Davis, James, Siegel, & Gould, 2005; Raybuck & Gould, 2009). However, the effects of chronic nicotine have yet to be investigated in a learned safety model. Moreover, considering PTSD is associated with nicotine dependence and heavy smoking (Cougle et al., 2010), investigating the effects of chronic nicotine on backwards trace conditioned safety also holds translational value. For example, it is unknown if chronic exposure to nicotine, such as experienced by smokers, exacerbates PTSD symptomology by disrupting learned safety. Therefore, based upon data showing an effect of acute nicotine on backwards trace conditioned safety, we performed a follow-up study using chronic nicotine administration. We predicted that if acute

nicotine alters safety learning via processes similar to that observed in acute nicotine's effects on hippocampus-dependent fear learning, that tolerance to nicotine's effects should occur in chronically exposed subjects.

Learned Safety

Learned safety is a cognitive process in which a cue becomes associated with the absence of an aversive event. As such, learned safety paradigms are contingent upon fear conditioning. More specifically, according to prediction error accounts of associative learning, in order for a CS to be learned as a safety cue it should fail to predict an expected aversive event (Schiller, Levy, Niv, LeDoux, & Phelps, 2008). Therefore, a safety cue gains opposing predictive strength and salience in reference to aversive fear learning and paradigms of learned safety require learning cues that indicate safety and danger.

Fear conditioning, a well-described model of fear learning, is a procedure that results in the association between an aversive US, for example a footshock, and CS such as a tone. However, this association is not limited to discrete cues and is found to also occur between a US and the conditioning context, a hippocampus-dependent learning processes (Kim, Rison, & Fanselow, 1993; Logue, Paylor, & Wehner, 1997; Phillips & LeDoux, 1992). As a result of fear conditioning, a neutral CS or context becomes learned as a conditioned exciter (CS+), due to its ability to unilaterally elicit outward expression of the learned fear association, i.e., freezing behavior. In contrast, a result of learned safety is that a CS learned as a conditioned inhibitor (CS-) of fear. Because a conditioned inhibitor of fear does not unilaterally elicit a behavioral response, but rather inhibits a response, a critical test for a

conditioned inhibitor is the summation test (Rescorla, 1969). During summation testing, a conditioned inhibitor (safety cue, CS-) is presented in compound with a conditioned exciter (danger cue, CS+). If safety is learned the subject will demonstrate reduced fear in response to the compound stimulus compared to presentation of the danger cue alone (Christianson et al., 2012; Kong et al., 2014). Therefore, summation testing was used in both behavioral assays, unpaired and paired (backward trace), which will be described and justified next.

Explicitly Unpaired Safety

Presentation of a cue explicitly unpaired with an aversive stimulus has been previously shown to result in robust learned safety (Pollak et al., 2008; Pollak, Monje, & Lubec, 2010). The procedure described herein involves presentation of a series of aversive footshocks (US) and unreinforced tone cues (CS-) using A+/AB- during training. Importantly, footshocks were not explicitly signaled with a discrete cue, thus the training context is associated with the aversive stimulus. As a result, trials in which footshock is delivered are described as A (Danger-Context) trials, while explicitly unpaired tone are designated as B (Safety-CS) trials (Figure 1).

As stated in Chapter 1, a number of studies have demonstrated that nicotine alters learning and memory (Davis, James, Siegel, & Gould, 2005; Gould & Wehner, 1999; Levin et al., 1990; Markou, 2008; Raybuck & Gould, 2009) and acute nicotine has been shown to enhance hippocampus-dependent contextual fear conditioning (Gould & Wehner, 1999). In contrast, acute nicotine was found to have no effect on auditory delay conditioned cued fear (Gould & Higgins, 2003), a hippocampus independent form of fear learning (Phillips & LeDoux, 1992). Considering that

nicotine specifically alters contextual fear learning, but both contextual and delay cued fear are amygdala dependent, these data strongly suggest that nicotine enhances cognitive processes peripheral to basic amygdala-related fear learning circuitry. Indeed, when nicotine was administered directly into the hippocampus mice showed similar enhanced contextual fear conditioning, but with no change in cued, hippocampus-independent, fear learning (Kenney, Raybuck, et al., 2012a). Further, nicotine was found to alter cell signaling cascades involved in long-term memory consolidation within the hippocampus, including the protein kinase A (PKA), extracellular signal-regulated kinase 1/2 (ERK1/2) (Gould et al., 2014). These data indicate that acute nicotine enhances hippocampal learning specifically, and that nicotine's effects on hippocampus-dependent fear learning are likely supported by recruitment of learning-related cellular signaling within the hippocampus.

While enhancement of contextual fear conditioning has been found to have no effect on learning discrete delay conditioned fear cues, little is known about how acute nicotine-associated changes in contextual learning might influence learning discrete safety cues. Considering that unpaired learned safety relies on contextual cues to indicate danger, we propose that acute nicotine-associated enhancement of contextual fear learning could result to altered learning of safety cues presented within the danger context. In support, prior work suggests that acute nicotine can disrupt fear inhibition by disrupting contextual fear extinction (Kutlu & Gould, 2014). Additionally, another report found that acute nicotine altered a discrete cued form of fear extinction. Specifically, animals treated with nicotine during fear

conditioning as well as extinction showed delayed cued extinction across six days (Elias, Gulick, Wilkinson, & Gould, 2010). The authors suggested that nicotine altered cue/context associations leading to a stronger cued fear response. Therefore, enhancement of contextual fear could lead to changes in learning co-occurring safety cues via context/cue second order associative learning process. This interpretation is also supported by other work, which indicates that context-cue stimuli may develop excitatory associations (Cunningham, 1981; Marlin, 1982; Pollak et al., 2010). As a result, we proposed that enhancement of the A+ (Context) could disrupt safety learning by potentiating context-cue associations. To assess the effects of acute nicotine on unpaired cued safety, summation testing occurred within the training context and subjects were presented with the unpaired CS. Thus, learning of safety was demonstrated if mice decreased fear in the presence of the safety cue within the training context (AB-) compared to the training context alone (A+).

Figure 1. Example of A+, AB- explicitly unpaired safety-learning procedure. A+ (Lightning Bolt) is the conditioning context and B- (Green) is a discrete CS presented within the context.

Backwards Trace Conditioned Safety

Presentation of a cue subsequent in time to an aversive event, backwards conditioning, has been shown to result in learned safety (Christianson et al., 2008; Mohammadi, Bergado-Acosta, & Fendt, 2014; Yarali et al., 2012). The procedure employed here, unlike the unpaired paradigm, is based on an A+/B- discrimination paradigm in which a signaled footshock (A+) is followed by an unreinforced tone presentation (B-) (Figure 2). Similar discrimination paradigms are well described and make good animal models of learned safety (Christianson et al., 2012). Further, discrimination paradigms have been used in clinical populations with PTSD (Jovanovic et al., 2012).

Figure 2. Example of A+/B-, backwards trace conditioned safety procedure. The A (red) cue is the conditioning discrete delay conditioned stimulus and the B (green) cue is a discrete trace conditioned stimulus.

A critical feature of trace conditioning is that the CS and US are presented in a temporally discontiguous configuration. Forward trace conditioning typically involves the presentation of a CS+ followed by a US after a trace interval and is hippocampus-dependent (Chowdhury, Quinn, & Fanselow, 2005; Quinn, Oommen, Morrison, & Fanselow, 2002). Moreover, when the US presented is aversive, forward trace conditioning results in the formation of a strong fear association with the CS. In particular, the dorsal hippocampus (DH) has been shown to be critical for

acquisition of trace fear conditioning (Chowdhury et al., 2005; Fendt, Fanselow, & Koch, 2005; Quinn, Loya, Ma, & Fanselow, 2005). As such, hippocampus-dependent mechanisms, related to declarative mnemonic processes, may act to bind together representations of the CS and US across the trace interval.

In contrast to forward trace fear conditioning, after backwards trace fear conditioning, where the CS is presented after the US, rats formed a weak fear association with the backwards CS (Quinn et al., 2002). This data suggests that reversed temporal ordering of stimuli results in the CS becoming a poor predictor of the US. However, backwards conditioned cues not only fail to predict the US, but after repeated training can predict the cessation of the US (Klopf, 1988). Thus, after repeated trials, backwards conditioning can result in the formation of a safety association with the backwards CS (Christianson et al., 2008; Gerber et al., 2014). Furthermore, the weak fear learning found in Quinn and colleagues also indicates that learned safety and fear processes work in opposition, which is consistent with learning models of conditioned inhibition (Rescorla, 1969; Williams, Overmier, & LoLordo, 1992). Importantly, formation of either a fear or safety association during backwards conditioning is partly mediated by the number of trials. Indeed, in the study by Quinn and colleagues, the training paradigm consisted of a single training session. Therefore, increased training is would be needed for backwards trace conditioning to result in a robust safety association.

Interestingly, while backwards trace conditioning results in weak fear learning, the mechanism mediating this fear association appears to be hippocampus-dependent. Indeed, pre-training lesions of DH abolish backwards

trace conditioning of fear (Quinn et al., 2002). This suggests that hippocampal processes, which appear to be necessary for forward trace fear conditioning, may also mediate backwards trace conditioned fear associations. Therefore, the hippocampus may be critical for binding excitatory fear CS-US associations across time under forward trace conditioning, but also during backwards trace conditioning. If this prediction is correct, it suggests that changes in hippocampal function that influence forward trace conditioning might also alter associations made during backwards trace conditioning. Such a change in trace conditioning could however be problematic, if an association between a backwards trace CS and US was enhanced, this danger association could compete with a more adaptive inhibitory safety association.

Work has demonstrated that acute nicotine enhances forward trace fear conditioning (Gould, Feiro, & Moore, 2004). Furthermore, nicotine locally infused into the DH replicates this effect (Raybuck & Gould, 2010). Therefore, nicotine could potentially modulate hippocampus-dependent associative learning, leading maladaptive enhancement of backwards trace conditioning fear association. Thus, mice were trained in an A+/B- backwards trace conditioning to assess the effects of acute and chronic nicotine on learned safety. Similar to the unpaired safety procedure, learning of safety was determined using summation testing. Unlike unpaired safety, animals were tested in an altered context, so that contextual cues would not influence behavior. During testing mice were assessed for freezing during presentations of A, (Light-Danger) and AB compound (Light/Tone-Safety).

Successful learning of conditioned inhibition was determined when animals froze less to the Light/Tone-Safety compared to the Light-Danger.

To determine if nicotine-associated changes safety learning observed during summation testing were the result of changes in excitatory trace fear learning, another set of animals was tested with presentation of the B tone (CS) in an altered context. This experiment used training similar to that described above. Critically, this experiment was used to assess if there was a shift in association, from safety to danger, in animals administered nicotine. Based on findings showing that acute nicotine disrupted backwards trace conditioned safety during summation testing, we hypothesized that mice administered acute nicotine would show enhanced freezing to the backwards trace CS.

Method

Subjects

Male C57BL/6 mice, aged 8 – 12 weeks old (Jackson Laboratory, Bar Harbor, ME) were used for all four experiments. Mice were housed in groups of four and maintained on a 12 hour light/dark cycle, food and water access was be *ad libitum*. All training and testing occurred between the hours of 9:00 am and 7:00 pm. Housing, behavioral, and surgical procedures were approved by the Temple University Institutional Animal Care and Use Committee.

Apparatus

For unpaired safety, training and testing occurred in four identical chambers (18 x 19 x 38 cm) contained within sound attenuating boxes (MED Associates, St.

Albans, VT). The sound attenuating boxes housed ventilation fans producing 69 dB background noise. An 85 dB white noise conditioned stimulus (CS) was produced using a speaker located on the wall of the conditioning chamber. 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 two second, 0.57 mA scrambled foot-shock.

Backwards trace conditioning occurred in the same chambers as in unpaired experiments. Testing occurred in a different room using four identical chambers housed within sound attenuating boxes (MED Associates, St. Albans, VT). 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 an programmable audio generator (MED Associates, ST. Albans, VT) via speakers housed within both the training and testing chambers. Additionally, a 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.

Behavioral Procedures

Unpaired Safety Learning

Freezing, defined as the absence of movement except for respiration, using a time sampling technique where each animal is observed for 1 second every 10 seconds, was used as the dependent measure. The unpaired safety conditioning

procedure is based on previous work by Pollak and colleagues (2010) and training consisted of three conditioning sessions over three days (Figure 1); each consisting of four explicitly unpaired US footshocks (2s, 0.57 mA) and CS white noise (85 dB) presentations over 11 minutes. Due to the explicit unpaired nature of the experiment, stimuli were segregated across time in an alternating fashion. For example, on Day 1, 4 footshock stimuli were presented, then after a time interval, 4 tone presentations were subsequently given. The order of presentation (shock then tone/tone then shock) was altered each training day. The interstimulus intervals for footshock and tone stimuli varied from 45 to 110 seconds. Additionally, the interstimulus interval separating the initial sequence of either US or CS from the second sequence was at least 88 seconds. Summation testing occurred 24 hours after the last day of conditioning in the same chambers as training. Testing consisted of two CS presentations (60s each) over 5 minutes (Figure 3).

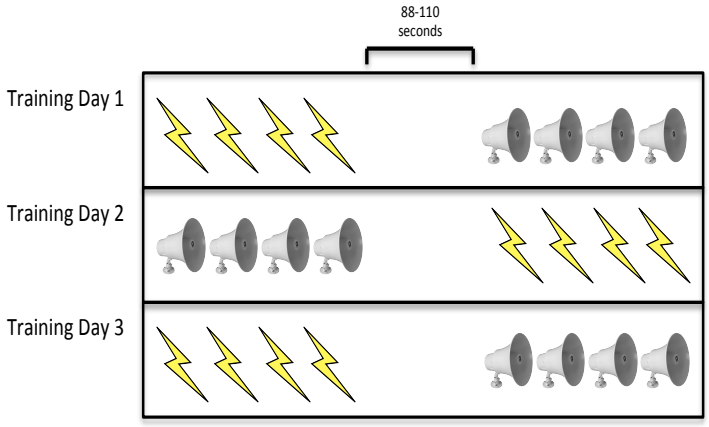

Figure 3. The conditioning protocol used for explicitly unpaired conditioned safety.

Backwards Trace Conditioned Safety

Similar unpaired safety, freezing was used to assess learning. Training consisted of three conditioning sessions occurring over the same number of days. Within each session, 5 signaled US footshocks (2s, 0.57 mA) were presented, followed by a 20 second trace interval (Figure 2). The footshock US was signaled by a delay CS+ (houselight, 30s) that co-terminated with the US. Following the trace interval a CS- (tone, 6K Hz, 85 dB) was presented. Each training session began with a 60s baseline period prior to the first trial. The intertrial interval was 90-120 seconds and pseudorandomly assigned. Summation testing occurred in an alternate context 24 hours after the last training session. Testing consisted of thee alternating presentations of light and light/tone compound (60s each), with 60 second intertrial intervals (Figure 4).

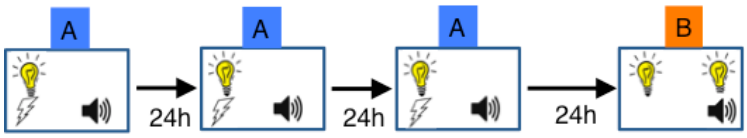

Figure 4. The conditioning protocol used for backwards trace conditioned safety.

Drug Administration and Experimental Design

The effects of acute and chronic nicotine on learned safety were examined. The dose of acute nicotine used for explicitly unpaired learned safety was 0.18 mg/kg. This dose was selected because it has been shown to enhance contextual fear conditioning and disrupt contextual safety discrimination and contextual fear extinction in C57BL/6J mice (Kutlu & Gould, 2014; Kutlu et al., 2014; Portugal, Wilkinson, Turner, Blendy, & Gould, 2012). Therefore, this dose was ideal to investigate whether acute nicotine disrupted unpaired safety by modulating learning of the context-danger association. The selected dose to investigate backwards trace conditioned safety, 0.09 mg/kg, has previously been shown to enhance forward trace fear conditioning in C57BL/6J mice (Gould et al., 2004; Raybuck & Gould, 2009). Therefore, this dose was ideal to assess acute nicotine's ability to disrupt backwards trace conditioned safety by altering the associative strength of the safety cue. Moreover, these doses of nicotine result in nicotine plasma levels similar to those found in smokers (Davis, James, Siegel, & Gould, 2005). Thus, these doses are physiologically relevant to smokers, and therefore results from these experiments may have translational value. For all four studies, nicotine hydrogen tartrate (Sigma, St. Louis, MO), reported as freebase weight, was dissolved in 0.9% sterile saline and was administered IP, 2 minutes prior to training and testing.

Considering that this study sought to investigate how nicotine impacts learned safety, it was critical that the doses selected did not intrinsically alter fear expression. Indeed, prior work indicates that the doses selected do not alter

hippocampus-independent (cued fear) forms of fear learning or expression (Carew & Wehner, 1999; Leach, Kenney, Connor, & Gould, 2015; Portugal, Wilkinson, Turner, et al., 2012; Raybuck & Gould, 2009). Thus, nicotine associated changes in learned safety may be interpreted as altered associative learning and not changes basic fear learning/expression.

Intraperitoneal (IP) injections were used for acute administration experiments as there is large body of work investigating the effects of acute nicotine on hippocampus-dependent cognition in which IP injections are used (Gould et al., 2004; Gould & Wehner, 1999; Kutlu & Gould, 2014). As a result, our results are comparable to a wide literature investigating the effects of acute nicotine on learning and memory. Also, prior studies have found that the effects of acute nicotine on hippocampus-dependent contextual and trace learning require administration prior to training and testing (Gould et al., 2004; Gould & Wehner, 1999). Therefore, nicotine was injected prior to training and testing sessions. In addition, for the chronic nicotine study, nicotine was delivered using subcutaneous osmotic minipump (Alzet) at 12.6 mg/kg/day for 14 days. This treatment schedule has previously been shown to model neural adaptations associated with chronic smoking, including upregulation of nicotinic receptors (Gould et al., 2012b; Gould, Wilkinson, Yildirim, Blendy, & Adoff, 2014). Considering that neural adaptations mediated by nicotinic acetylcholine receptors (nAChRs) are thought to underlie nicotine addiction, this administration strategy is a good model of the pharmacological effects of continuous nicotine consumption similar to that of smokers.

Surgery

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). Osmotic minipumps were surgically implanted subcutaneously via an incision posterior to the scapulae. The incision site was closed with surgical staples. Minipumps delivered chronic saline or nicotine (12.6 mg/kg/d) for 14 days (testing occurring on the 14th day).

Data Analysis

For all four experiments freezing data were obtained using a time sampling method as described previously (Poole, Connor, & Gould, 2014). Freezing data was transformed into percent time freezing. Additionally, results were considered significant at $p \leq 0.05$. All data are presented as means ± SEM. Statistical analysis was performed using SPSS 16.0.

Explicitly Unpaired Safety. Freezing data was collapsed into Danger-Context and Safety-CS presentations. A mixed-design ANOVA compared Danger-Contextual vs. Safety freezing (within-groups comparison) and saline vs. nicotine (between group comparison). Two animals were removed from analysis due to freezing levels exceeding 2 standard deviations from the mean.

Backwards Trace Conditioned Safety. Freezing data was analyzed using a mixed-design ANOVA to compare freezing to the Light-Danger cue and the compound Light/Tone-Safety. A priori planned comparisons paired samples *t*-tests where performed to compare within subjects change in freezing during testing conditions. Between subjects *t*-tests were used to analyzed Pre-CS freezing.

Freezing was scored for the entire duration and data was collapsed into Light-Danger and Light/Tone-Safety conditions for statistical analysis.

Backwards Trace Conditioned Fear. Similar to previous work (Poole, Connor, & Gould, 2014; Raybuck, Portugal, Lerman, & Gould, 2008), a one-way ANOVA was used to analyze freezing behavior for Pre-CS and an additional one-way ANVOA was used to analyze freezing during presentation of the CS. Freezing was scored for the entire duration of testing during the first 3 minutes (Pre-CS) and the latter 3 minutes (CS). One animal was removed from analysis due to freezing over 2 standard deviations from the mean.

Results

The effects of acute nicotine on unpaired learned safety

To examine learned safety, a summation test was used in which mice were re-exposed to the training context and then presented with two iterations of the previously unpaired CS. Learning of safety therefore is demonstrated by decreased freezing during CS (safety cue) presentations compared to context exposure alone. Therefore, we sought to examine the data for changes in freezing due to testing condition and drug administration. A mixed-design ANOVA indicated a significant within subjects main effect of testing condition (Danger-Context vs. Safety-CS), $F_{(1, 24)} = 77.956$, $p < 0.001$. Additionally, a significant between subjects main effect of Drug (Saline vs. Nicotine), $F_{(1, 24)} = 8.793$, $p = 0.007$ was observed, but no significant interaction was found (Condition x Drug), $F_{(1, 24)} = 0.188$, $p = 0.669$ (Figure 5).

The significant main effect of testing condition indicates that both drug groups learned safety. Specifically, mice treated with saline froze less during Safety-CS (M = 37.18%, SD = 19.72%) compared to Danger-Context condition (M = 64.10%, SD = 19.33). Mice treated with nicotine showed as similar pattern of behavior, freezing less during Safety-CS (M = 85.47%, SD = 13.52%) than Danger-Context (M = 55.77%, SD = 22.41%). In addition, the significant main effect of drug suggests that nicotine increased freezing during both testing conditions. Freezing during Context-Danger phase was higher in the nicotine treated group (M = 84.12%, SD = 13.93%) compared to the saline group (M = 64.10%, SD = 19.33%), as well as during the Safety-CS phase (Nicotine: M = 54.76%, SD = 21.86; Saline: M = 37.18%, SD = 19.72%). Thus, animals treated with acute nicotine showed increased freezing in response to the Context as well to Tone-Safety presentations. However, while nicotine treatment increased freezing it did not alter summation testing of learned safety. We interpret this finding to suggest that nicotine leads to enhancement of the contextual/danger association, leading to the observed upward shift in freezing to the safety cue.

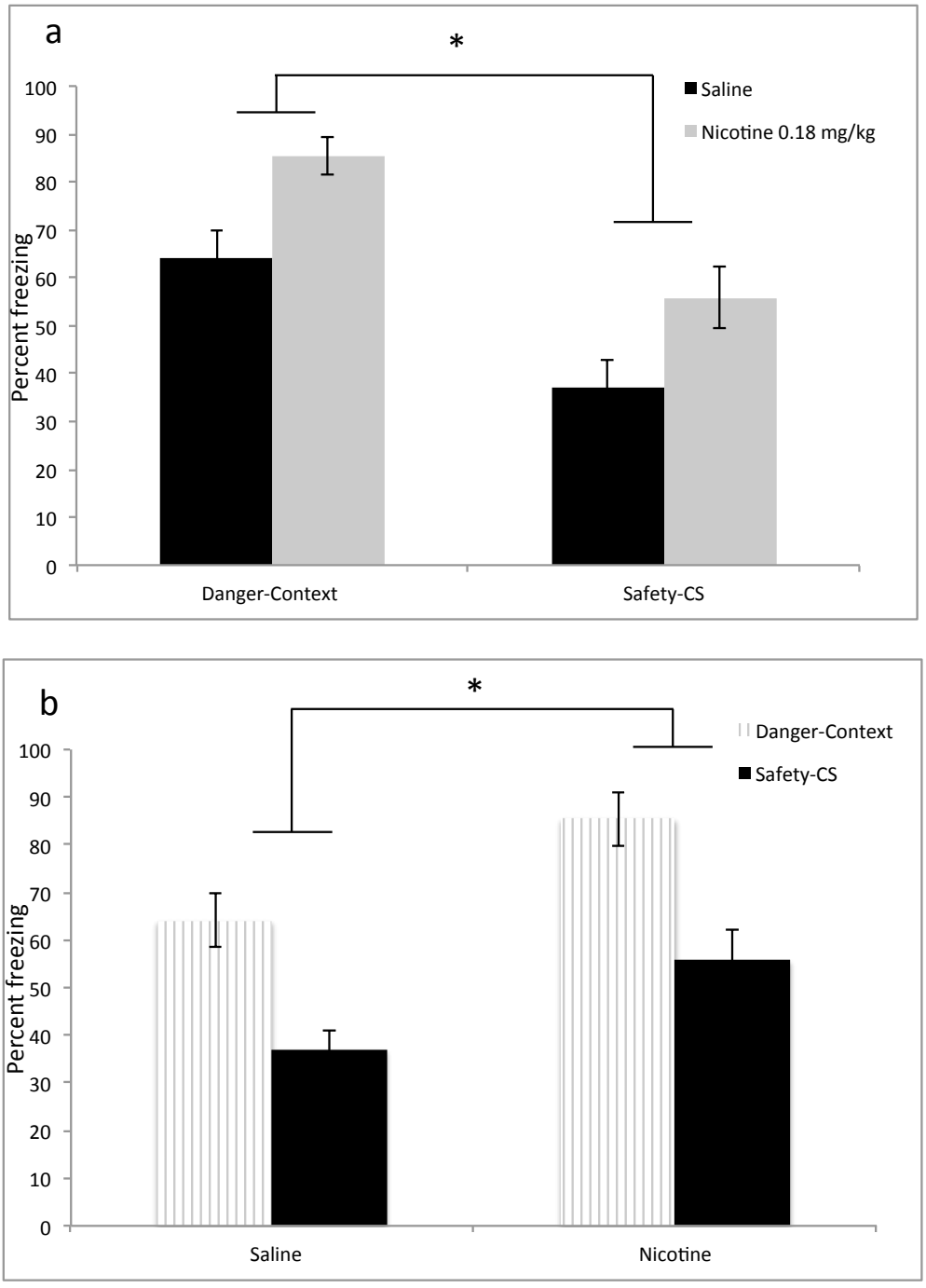

Figure 5: Explicitly Unpaired Safety. (a) Main effect of Condition, mice showed significantly less freezing during presentation of explicitly unpaired cue (Safety-CS) compared to the training context alone (Danger-Context). (b) Main effect of Drug, mice treated with nicotine showed increased freezing compared to saline treated mice. No significant Drug x Condition interaction suggests that nicotine did not disrupt conditioned inhibition of fear, i.e., learned safety. Error bars indicate SEM, (n = 13), (*) indicates main effects ANOVA, $p < 0.05$

The effects of acute nicotine on backwards trace conditioned safety

A mixed-design ANOVA found a significant interaction (Drug x Condition) $F_{(1,16)} = 7.910$, $p = 0.013$. No within subjects main effect of testing condition (Danger vs. Safety) $F_{(1,16)} = 0.963$, $p = 0.341$, but a between subjects main effect of drug (Saline vs. Nicotine) was observed $F_{(1,16)} = 8.209$, $p = 0.011$. Planned comparison paired *t*-tests indicated that saline treated control animals froze less during the Light/Tone-Safety (M = 29.02%, SD = 14.64%) compared to Light-Danger (M = 46.91%, SD = 17.59%), $t(8) = 2.42$, $p = 0.042$. Therefore, mice treated with saline showed learned safety. In contrast, animals treated with nicotine showed no difference in freezing Light-Danger (M = 46.91%, SD = 9.66%) vs. Light/Tone trials (M = 55.55%, SD = 13.03%), $t(8) = 1.474$ $p = 0.179$. Finally, an independent-samples *t*-test found no difference between saline (M = 12.96%, SD = 20.03%) and nicotine (M = 12.96%, SD = 18.21%) treatment during Pre-CS baseline period $t(8) = 0.0$ $p = 1.0$. Thus, groups showed no differences in non-associative freezing behaviors (Figure. 6).

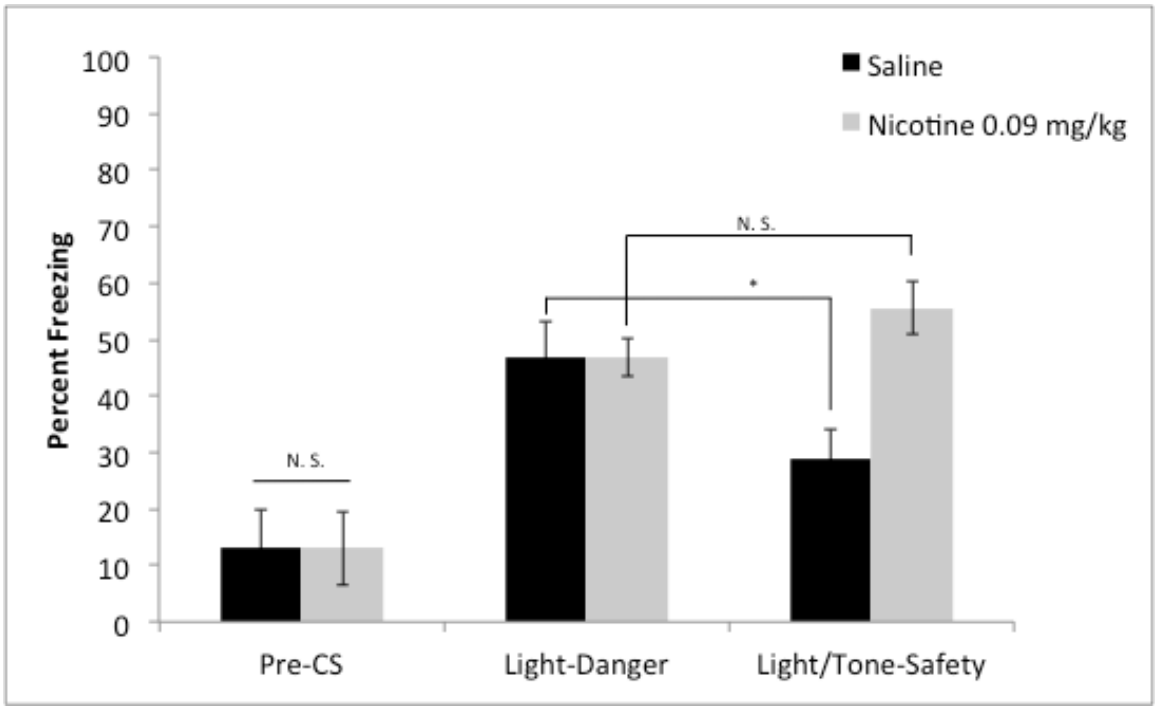

Figure 6: Backwards Trace Conditioned Safety, saline treated mice showed significantly less freezing during presentation of light/tone compared to light alone. Mice treated with nicotine failed to show learned safety with freezing similar between danger and danger/safe compound. Error bars indicate SEM, (n = 9), (*) indicates paired samples *t*-test, $p < 0.05$. A independent samples *t*-test found no significant difference in Pre-CS freezing (Saline vs. Nicotine), $p > 0.05$.

The effects of chronic nicotine on backwards trace conditioned safety

To assess if chronic nicotine might also disrupt learned safety we performed the same backwards 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 (Danger vs. Safe) $F_{(1,28)} = 31.276$, $p < 0.001$, but no significant between subjects main effect of drug (Nicotine vs. Saline) $F_{(1,28)} = 0.664$, $p = 0.422$ or interaction was observed (Condition x Drug) $F_{(1,28)} = 0.414$, $p = 0.525$. 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 to 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 = 28.81%, SD = 15.07%) compared to the Light-Danger (M = 42.36%, SD = 13.43%) (Figure 7). Finally, an independent samples *t*-test found that nicotine treated mice froze significantly more during the Pre-CS period compared to saline treated mice $t(28) = 3.002$, $p = 0.006$. Because means indicate that nicotine treated mice showed fear learning, by freezing at greater levels during Light-Danger (M = 42.36%, SD = 13.43%) compared to Pre-CS (M = 25%, SD = 27.51%), differences in Pre-CS freezing did not alter our interpretation of the mixed ANOVA results.

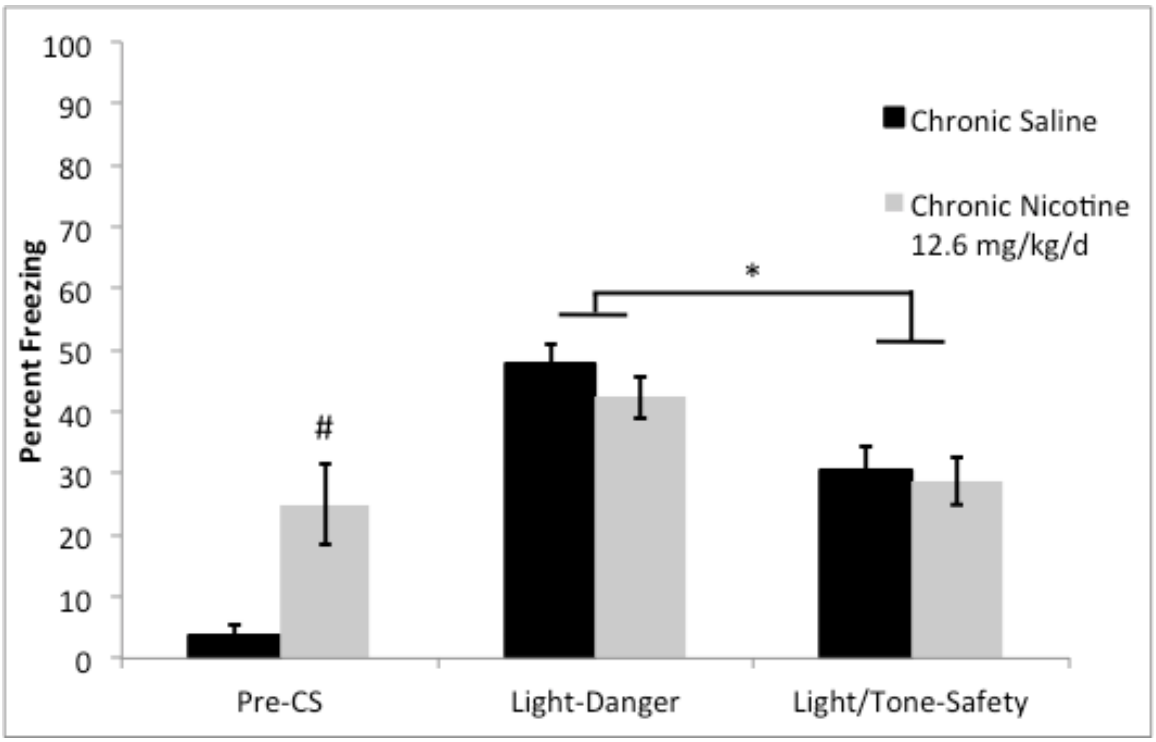

Figure 7: Backwards Trace Conditioned Safety, saline and nicotine treated mice froze less during presentation of Light/Tone-Safety compared to Light-Danger. Error bars indicate SEM, (n = 14-16), (#) indicates significant between subjects *t*-test during Pre-CS (Saline vs. Nicotine), $p < 0.05$. (*) indicates significant main effect of testing condition (Light-Danger vs. Light/Tone-safety), $p < 0.05$.

The effects of nicotine on backwards trace fear conditioning

In order to assess whether acute nicotine disrupts backwards trace conditioned safety by enhancing a fear association with the backwards CS, an alternate test was implemented. Rather than a summation test, mice were placed in an alternate context and presented with the backwards CS alone after a 3 minute Pre-CS period. A one-way ANOVA found no significant effect of drug (Saline vs. Nicotine) during the Pre-CS period $F_{(1,13)} = 0.021$, $p = 0.888$; saline (M = 8.73%, SD = 12.36%) and nicotine (M = 9.72%, SD = 14.16%). This null finding suggests that acute nicotine had no effect on non-associative or generalized freezing. However, a

one-way ANOVA found a significant effect of drug (Saline vs. Nicotine) during the CS $F_{(1,13)} = 8.960$, $p = 0.01$. Importantly, as hypothesized, nicotine treated mice froze more during presentation of CS (Tone) (M = 29.16%, SD = 14.47%) than saline treated mice (M = 10.31%, SD = 8.74%) (Figure 8). Therefore, nicotine treated mice showed enhanced freezing behavior in response to CS.

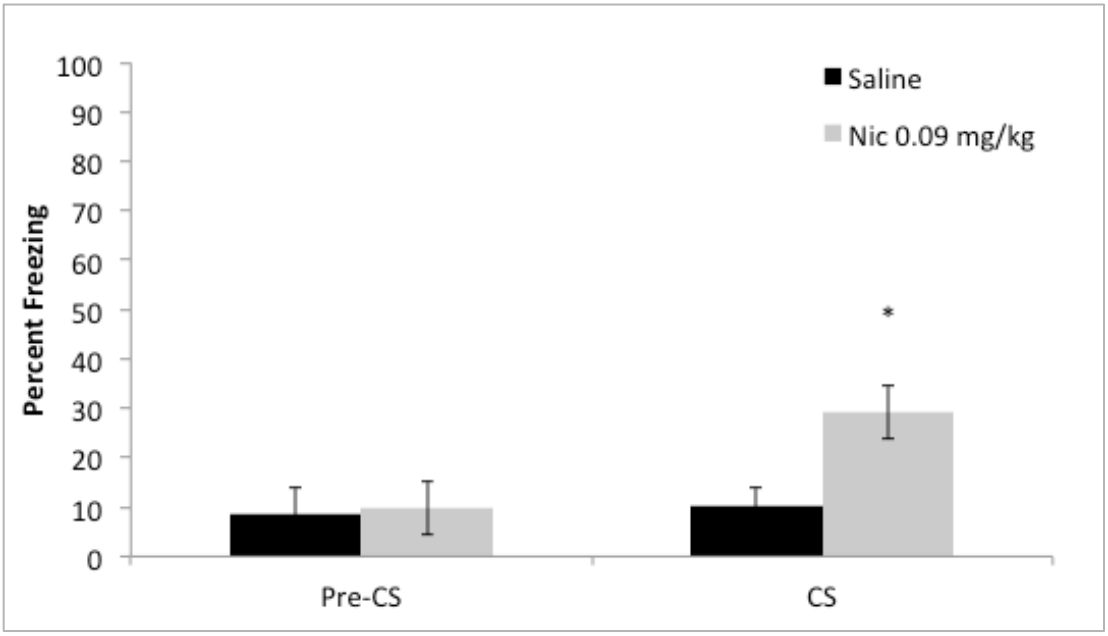

Figure 8: Backwards Trace Fear Conditioning, mice did not show any difference in freezing during the Pre-CS condition indicating no differences in non-associative or generalized freezing. In contrast, nicotine treatment resulted greater freezing to the CS compared to saline treatment, indicating the formation of a fear association with the backwards trace CS after nicotine treatment. Error bars indicate SEM, (n = 7-8), (*) indicates significant one-way ANOVA, $p < 0.05$.

Discussion

This study was the first to examine the effects of nicotine on learned safety. Here, it was hypothesized that nicotine could alter learned safety by enhancing contextual and backwards trace fear associations. First, using an explicitly unpaired safety procedure, we investigated if acute nicotine-associated enhancement of contextual fear learning could interfere with learning a discrete safety CS within the same context. Summation testing indicated no effect of acute nicotine on cued safety learning in the unpaired preparation. Secondly, using a backwards trace conditioning procedure; we assessed the effects of acute and chronic nicotine. Summation testing revealed that acute nicotine administration disrupted safety, but chronic nicotine did not. Finally, we observed that acute nicotine enhanced the formation of a fear association with the backwards trace CS. This data strongly suggests that disrupted safety learning observed in the backwards trace conditioned paradigm is the result of potentiation of maladaptive fear learning of the backwards CS.

Explicitly Unpaired Safety

Acute nicotine administration has been shown to enhance contextual fear learning (Davis, Kenney, & Gould, 2007; Gould & Higgins, 2003) and can disrupt fear inhibition (Kutlu & Gould, 2014; Kutlu et al., 2014). As a result, we hypothesized that nicotine would disrupt learning a discrete safety cue when presented within a danger-associated context. Consistent with previous findings from Pollak and colleages, (2010), we found that an explicitly unpaired CS presentation resulted in learned safety in saline control mice during testing after

three days of training. Similarly, although contrary to our hypothesis, acute nicotine treated mice also demonstrated learned safety, showing reduced fear behavior during presentation of a safety cue during summation testing. Therefore, we conclude that acute nicotine treatment had no effect on learned safety, as defined by the ability of the CS to reduce freezing to the training context. As expected acute nicotine treatment resulted in significantly greater freezing to the training context in agreement with previous work (Gould & Wehner, 1999; Portugal, Wilkinson, Turner, et al., 2012). These data suggest two important findings, first that acute nicotine, at a dose that enhances contextual fear learning, does not intrinsically disrupt learned safety. Secondly, enhancement of context-fear learning by acute nicotine is not sufficient to interfere with cued safety learning. Finally, while learning of safety was intact in both groups, mice administered nicotine demonstrated overall increased in freezing behavior during both testing conditions.

While the data suggests that acute nicotine does not block learning of safety, acute nicotine administration resulted in increased freezing during both testing conditions (Danger-Context and Safety-CS). This finding is consistent with prior work indicating that experimental manipulations that change the strength of contextual learning can modify the expression of learned cue associations via context-cue associative learning (Marlin, 1982). Interestingly, while nicotine may not interfere learning safety learning per se, this shift in fear during presentation of the safety cue may have clinical implications for individuals at risk of PTSD. For example, individuals with PTSD show disrupted emotional regulation, including

potentiated fear responses and hyper-arousal in response to contextual threat (Jovanovic, Norrholm, Blanding, Phifer, et al., 2010; Pole et al., 2009). Thus, acute nicotine-associated enhancement of contextual fear could result in hyper-arousal even if safety cues are learned. Moreover, acute nicotine may result in a state in which cues that indicate safety are less effective at reducing fear in dangerous contexts, because of enhanced contextual associations.

For this initial study the role of the hippocampus was not directly investigated, however our findings appear consistent with prior work suggesting that acute nicotine acts specifically on hippocampal forms of learning. For example, acute nicotine enhances contextual fear learning, which is hippocampus-dependent, but does not alter hippocampus-independent delay cued fear conditioning (Corcoran & Maren, 2001; Gould & Higgins, 2003; Kim et al., 1993; Phillips & LeDoux, 1992). Thus, increased freezing, observed during summation testing, is consistent with changes in hippocampus-dependent context learning processes. In contrast to contextual learning, the role the hippocampus plays in forming safety associations with unpaired cues is not as well understood (Fanselow, 2010; Pollak et al., 2008). Considering acute nicotine did not disrupt unpaired learned safety, our results suggest the possibility that learned safety is not necessarily hippocampus-dependent. Unpaired safety could possibly be learned via hippocampus-independent processes, similar to delay cued fear conditioning. In support, prior work found that pre-training lesions of the hippocampus did not block learning of safety (Heldt, Coover, & Falls, 2002). Thus, hippocampal processes may not be necessary to encode and express learned safety. Alternatively, learned

safety may be processed in parallel by redundant mechanisms. Thus, further work is needed to explicitly examine the role of the hippocampus in unpaired learned safety.

Backward Trace Conditioning

Acute nicotine has been shown to enhance hippocampal forms of fear learning, including trace fear conditioning (Gould et al., 2004; Gould & Wehner, 1999). Therefore, the effects of acute nicotine on backwards trace conditioned safety paradigm were investigated. The present study found that acute nicotine, at a dose that enhances forward trace conditioning, disrupted learned safety. The disruption of learned safety was observed as a failure to reduce freezing behavior in the presence of the backwards CS during summation testing. In contrast, animals treated with saline showed a reduction in freezing when presented with a backwards CS, indicating formation of a safety association. Critically, a follow up experiment showed that acute nicotine facilitates a fear association with the backwards CS. Thus, the effects of acute nicotine on backwards trace conditioned safety stem from enhanced trace fear learning. In sum, acute nicotine causes the formation of a maladaptive fear association with a backwards trace CS, which control animals learn as a safety cue.

Within the context of prior work investigating the effects of acute nicotine on learning, the finding that acute nicotine disrupts associative learning appears initially divergent. For example, while withdrawal from chronic nicotine results in cognitive deficits, acute nicotine has been repeatedly shown to enhance hippocampus-dependent forms of learning (Carew & Wehner, 1999; Davis et al.,

2005; Kenney, Adoff, Wilkinson, & Gould, 2011). However, recent findings suggest that acute nicotine may disrupt fear inhibition when hippocampus-dependent learning is engaged during formation of fear associations. For example, acute nicotine has been found to disrupt contextual fear discrimination and contextual fear extinction (Kutlu & Gould, 2014; Kutlu et al., 2014). We propose that a fundamental mechanism underlying our observed acute nicotine-associated disruption in backwards trace conditioned safety is the formation of a maladaptive backwards trace CS fear association. Moreover, that the mechanism that acute nicotine acts on to prevent backwards trace safety may be a common mechanism mediating acute nicotine-associated enhancement of forward trace fear conditioning. In support, the effects of acute nicotine on fear learning have been shown to be specific to hippocampus-dependent forms of learning and hippocampal processes are critical for learning temporally discontiguous stimulus associations, as in trace conditioning (Bangasser, Waxler, Santollo, & Shors, 2006; Gould et al., 2004; Gould & Wehner, 1999). Indeed, when we tested for a fear in response to the backwards trace CS, animals treated with acute nicotine showed enhanced fear in the novel testing context. This behavioral result strongly supports the hypothesis that acute nicotine prevents safety by facilitating an association between the backwards paired US and CS. Therefore, it appears the effects of acute nicotine administration on trace learning occur independent of temporal ordering of stimuli. One possibility is that acute nicotine may induce a more permissive state for associative memory to occur, which would also be sufficient to explain acute nicotine-associated phenomena in backwards and forward fear trace fear

conditioning paradigms. In direct contrast to animals that received acute nicotine, freezing levels to the CS in the novel context in control mice were low and consistent with the formation of an inhibitory association (Rescorla, 1969). In sum, acute nicotine can facilitate an association between the backwards trace CS and footshock, leading to the formation of an enhanced US-CS association and this danger association leads to disruption of learned safety by altering the inhibitory strength of the safety cue.

In contrast to the effects of acute nicotine, we observed no effect of chronic nicotine on backwards trace conditioned safety. This null finding is in agreement with prior studies showing that while initial nicotine exposure leads to changes in hippocampal learning, chronic administration results in tolerance to such cognitive effects (Davis et al., 2005; Portugal, Wilkinson, Kenney, Sullivan, & Gould, 2012). Tolerance to cognitive effects of nicotine is thought to be mediated by neural adaptations induced by continuous nicotine exposure, including desensitization and upregulation of nAChRs (Marks et al., 1992; Marks, Grady, & Collins, 1993). Behavioral work supports this, as the rate at which cognitive tolerance develops has been shown to temporally align with nAChR upregulation within the hippocampus (Gould, Wilkinson, Yildirim, Blendy, et al., 2014). Additionally, acute nicotine has been shown to enhance trace fear conditioning, while chronic nicotine did not (Raybuck & Gould, 2009). Therefore, the lack of an effect of chronic nicotine on backwards trace conditioned safety is also consistent with our interpretation that acute nicotine facilitates a maladaptive backwards trace US-CS association.

The mechanisms underlying the effects of nicotine on hippocampus-dependent learning processes is still being uncovered. However, it is known that nAChRs are richly expressed within the hippocampus and activation of these receptors can modulate long-term plasticity (LTP), a process underlying learning-dependent neural adaptations (Ge & Dani, 2005; Marks, Romm, Campbell, & Collins, 1989; Marks et al., 1992). For example, nicotine has been shown to potentiate hippocampal LTP and activation of nAChRs reduced the threshold for LTP induction within the hippocampus (Ge & Dani, 2005; Sawada, Yamamoto, & Ohno-Shosaku, 1994). Therefore, nicotine likely acts on physiological substrates of cognition within the hippocampus. In support, nicotine infused into the hippocampus was sufficient to enhance trace fear learning and genetic deletion of β2 containing nAChRs prevented acute-nicotine enhancement of trace fear conditioning (Davis & Gould, 2007; Raybuck & Gould, 2010). Therefore, Chapter 3 describes studies that investigated brain regions, including the hippocampus, which may be sufficient to mediate the effects of acute nicotine on backwards trace conditioned safety.

Conclusion

Overall, the present data suggests that acute nicotine can disrupt learned safety in a paradigm dependent manner. Specifically, we found that acute nicotine administration can disrupt learned safety by altering the associative strength of a backwards trace CS. Indeed, conditioned inhibition of fear involves competing associations between conditioned excitation (CS+) and conditioned inhibition (CS-)

(Rescorla, 1969). Therefore, changes to learning processes that facilitate the formation of a CS+ association with a cue, may inherently disrupt forming a CS- association with the same cue. Moreover, our data showing nicotine-dependent formation of a CS+ (fear) association with a backwards CS strongly suggests that acute nicotine results in a neural state more permissive to forming CS-US associations regardless of temporal order. This finding has implications for our understanding of the relationship between cognition, PTSD, and smoking. For example, initiation of smoking under stressful conditions may lead to an inability to properly learn and respond to cues that indicate safety. Moreover, nicotine could also lead to a situation in which cues that should be learned as safe are actually learned as dangerous. Thus, failure to form adaptive safety associations that inhibit fear could exacerbate dysfunctional emotional regulation in trauma-exposed individuals. In these ways acute nicotine may result in maladaptive learning and behavioral responses to stressful conditions and could precipitate or exacerbate symptoms associated with PTSD.

A substantial link has been established between PTSD and nicotine consumption, however the nature of this relationship is not well understood (Cougle et al., 2010). Previous work has shown that acute nicotine can potentiate some forms of emotional associative learning such as fear learning. Thus, an important consideration is that enhanced learning may not necessarily be adaptive. For example, acute nicotine-associated enhancement of contextual fear learning and delay of contextual fear extinction may exacerbate formation and maintenance of context related trauma associations (Kutlu & Gould, 2014). Further, we show

here that adaptive learning of backwards safety is disrupted by facilitation of US-CS associative learning. Thus, changes in associative memory may be an important component in the role of smoking and the development and/or maintenance of PTSD symptoms by making cues that indicate safety less salient or effective. Considering that disrupted safety learning has been identified as a possible biomarker for PTSD (Jovanovic et al., 2009; Jovanovic, Norrholm, Blanding, Davis, et al., 2010), nicotine-associated changes in learned safety may facilitate emotional dysregulation.

Finally, our results indicating that acute nicotine alters backwards trace, but not unpaired conditioned safety, supports a more general view that nicotine has effects on specific forms of learning and memory. Specifically, acute nicotine has been shown to alter hippocampus-dependent associative learning, but not hippocampus-independent learning (Gould & Higgins, 2003; Kenney et al., 2011; Portugal, Wilkinson, Turner, et al., 2012). These findings suggest that acute nicotine may have little direct effects on emotional processes, but rather acts on associative learning processes that are engaged by emotional learning under certain conditions, such as when stimuli are temporally discontiguous, i.e., trace conditioning. Thus, nicotine may modulate emotional processing indirectly by altering higher-order hippocampal learning process. This interpretation of acute nicotine-associated effects on learning is also parsimonious with our results, in which acute nicotine did not disrupt unpaired safety. For example, we observed enhanced contextual fear learning, which increased fear responding, but did not disrupt formation of a safety association with a discrete unpaired CS. As stated

previously, acute nicotine has been shown to have no effect on formation of fear associations with discrete cues that temporally overlap with aversive stimuli (delay conditioning) (Davis & Gould, 2007). Considering our findings in unpaired learned safety, this suggests that discrete unpaired cues may be learned in a hippocampus-independent fashion, similar to delay conditioned cues. While further work is necessary to better understand how unpaired cues are learned, unpaired cues may be learned similar to delay conditioned cues because they overlap in time with unreinforced periods during conditioning. Indeed, temporal overlap is a critical factor in determining whether associative learning is hippocampus-dependent or independent, i.e., delay vs. trace conditioning.

In sum, acute nicotine completely prevented normal fear inhibition during summation testing after backwards trace conditioning. Considering that acute nicotine, even at a dose sufficient to alter hippocampus-dependent contextual learning, did not block unpaired safety learning, we suggest that acute nicotine does not inherently prevent learned safety. Rather, acute nicotine can disrupt backwards trace conditioned safety by facilitating a maladaptive backwards trace fear association.

CHAPTER 3

REGION SPECIFIC EFFECTS OF NICOTINE ON BACKWARDS TRACE CONDITIONED SAFETY

Rationale

In Chapter 2, we found that treatment with acute nicotine resulted in disrupted backwards trace conditioned safety. Importantly, we also showed that acute nicotine enhanced formation of a fear association with a backwards trace CS. Thus, the effect on backwards trace conditioned safety appeared to result from the formation of a maladaptive fear association with the backwards trace CS. Specifically, acute nicotine resulted in a danger-CS (CS+) association and this shift in valence explains nicotine-associated disruption of the backwards trace safety-CS (CS-) association. While the effects of acute nicotine on associative fear learning and brain regions underlying acute nicotine-associated effects on cognition have previously been studied (Kenney, Raybuck, & Gould, 2012; Raybuck & Gould, 2010), the brain regions involved in acute nicotine-associated effects on backwards conditioned safety have yet to be investigated. Because previous research has shown that nicotine enhances DH and medial prefrontal cortex (mPFC) dependent learning (Kenney, Raybuck, & Gould, 2012b; Raybuck & Gould, 2010), we hypothesized that direct effects of nicotine in the DH and mPFC may also facilitate maladaptive learning backward trace fear learning.

The hippocampus and mPFC where identified as target regions, as these neural substrates have been shown to critical for forward trace conditioning

(Chowdhury et al., 2005; Gilmartin & McEchron, 2005). As discussed previously, in contrast to backwards conditioning, forward trace conditioning is typified by presentation of a CS followed by a temporally discontiguous US, which results in an association between CS and US (CS+). Importantly, unique cognitive and neural processes are recruited in order to bridge this temporal gap, facilitating an association between the CS and US (Connor & Gould, submitted 2016; Gilmartin, Kwapis, & Helmstetter, 2013; Jonathan Raybuck & Lattal, 2014; Shors, 2004; Woodruff-Pak & Disterhoft, 2008). For example, working memory and declarative memory processes appear to be recruited for acquisition of forward trace conditioning (Carter, Hofstötter, Tsuchiya, & Koch, 2003; Connor & Gould, submitted 2016; Squire & Zola, 1996). Based on our results indicating that acute nicotine enhances the fear association between the US and CS during backwards conditioning, we hypothesize that nicotine disrupts backwards trace conditioned safety by acting on neural substrates that also mediate forward trace conditioning, leading to a more labile state for the formation of trace associations.

Rationale for Regions of Interest

Hippocampus

The hippocampus is a forebrain region critical for encoding and storage of some forms of long-term associative memory (Fanselow & Dong, 2010; Squire & Zola, 1996). Moreover the hippocampus plays a role in learning temporally non-overlapping sequences (Honey, Watt, & Good, 1998) and is critical for forward trace conditioning (Bangasser et al., 2006; Moyer, Deyo, & Disterhoft, 1990; Shors,

2004). There is high expression of nAChRs within in the hippocampus and nAChRs are important for learning-related neural plasticity within hippocampus (Fujii, Jia, Yang, & Sumikawa, 2000; Guan, Nakauchi, & Sumikawa, 2006; Matsuyama, Matsumoto, Enomoto, & Nishizaki, 2000; Placzek, Zhang, & Dani, 2009). For example, small alterations in the timing of nAChR activation determines stability of changes in synaptic efficiency (Ge & Dani, 2005). Thus, nicotine administration could alter learning of backwards conditioned stimuli by reducing the threshold for plasticity underlying the formation of maladaptive memory engrams.

The hippocampus has been shown to be important for the effects of acute nicotine on learning and memory (Kenney, Raybuck, & Gould, 2012b; Raybuck & Gould, 2010). However, the DH and ventral hippocampus (VH) are functionally and genetically divisible regions (Fanselow & Dong, 2010). Fanselow and Dong (2010) suggest that the DH is important for cognitive processing, e.g., contextual and spatial learning, while the VH is preferentially involved in anxiety and emotional processing. Moreover, behavioral studies have shown that nicotine has divergent cognitive effects when directly infused into the DH and VH. For example, nicotine infused into the DH potentiates, but VH administration disrupts, forward trace fear conditioning (Raybuck & Gould, 2010). This suggests nicotine may modulate hippocampal processing necessary for trace learning. However, it is not clear what role the hippocampus plays in modulating the processing of discontiguous stimulus presentation when the CS-US relationship is reversed. In the previous chapter we showed data indicating that backwards trace conditioning results in the formation of a safety association, but it is unknown if direct administration of nicotine into the

DH would interfere with this safety learning process by facilitating a fear association with the backwards trace CS. To this end, we used direct infusion of nicotine into the DH to investigate this.

Considering that learned safety modulates fear expression and innate anxiety (Pollak et al., 2008), the VH was also selected as a targeted for direct infusion of nicotine. This decision was supported by work indicating that the VH may be important for expression of fear (Kjelstrup et al, 2004). Previously, nicotine infused into the VH was found to disrupt hippocampus-dependent learning (Kenney et al., 2012; Raybuck & Gould, 2010). Therefore, an alternative hypothesis is that acute nicotine-associated disruption of backwards trace safety is caused by deficits in hippocampal memory. Moreover, the VH sends direct projections to the amygdala, therefore changes in VH processing may influence amygdala-dependent fear expression (Christianson et al., 2008). While Raybuck and Gould (2010) found DH nicotine administration enhanced trace fear conditioning, the number of trials and length of training in that preparation was lower than described here for the backwards trace conditioned safety assay. As a result, increased conditioning trials in backwards trace conditioned safety paradigm might elicit stress related neural adaptations within the hippocampus, which shift cognitive processing from DH to VH. Evidence suggests that under baseline conditions the DH has a lower threshold for long-term potentiation (LTP) compared to VH, which may be related to the importance of the DH in cognition. However, stress can shift this asymmetry in the opposite direction (Maggio & Segal, 2009). Therefore, a greater number of trials and conditioning days required for backwards trace could lead to increased stress

levels. Therefore, we explicitly investigated the effect of nicotine infused into the VH to assess for changes in backwards trace conditioned safety.

Prefrontal Cortex

Cholinergic signaling also modulates processing within cortical regions (Everitt & Robbins, 1997). In addition, nAChRs are widely expressed within the mPFC and participate in cognitive functions including attention and working memory (Guillem et al., 2011; Sarter, Parikh, & Howe, 2009). Moreover, nicotine administration has direct effects on working memory performance (Levin & Torry, 1996; Levin & Rose, 1991), and nicotine directly administered to the mPFC was shown to enhance trace fear conditioning (Raybuck & Gould, 2010). This is consistent with work showing that trace conditioning recruits activation of mPFC via working memory processes (Gilmartin, Kwapis, & Helmstetter, 2012). Thus, these data suggest that nicotine may readily alter working memory processes mediated by the mPFC via nAChRs during trace conditioning. However, it is unknown if nicotine within the mPFC might alter learning in a backwards trace conditioning paradigm.

Since the prelimbic region (PL) of the mPFC receives thalamic and hippocampal inputs, and is recruited for working memory tasks (Hoover & Vertes, 2007; Ragozzino, Detrick, & Kesner, 2002), it may play an important role in associative learning when stimuli are discontiguous. In support, during trace fear conditioning, neurons within the PL showed sustained firing during the trace interval between CS and US presentations (Gilmartin & McEchron, 2005). In addition, optogenetic inactivation of the PL disrupts acquisition of trace fear

conditioning (Gilmartin, Miyawaki, Helmstetter, & Diba, 2013). Thus, the mPFC may act to bridge the temporal gap during forward trace conditioning facilitating a CS-US trace memory. We have shown that backwards trace conditioning leads to an inhibitory (CS-) safety association with the backwards CS, but nicotine prevents this. Therefore, it is possible nicotine acts on the mPFC to alter working memory process leading bridging of the backwards trace interval. Such a mechanism could explain formation of a danger-CS association that appears to cause disruption of learned safety. As a result, we investigated the effect of direct administration of nicotine into the mPFC on backwards trace conditioned safety.

Method

Subjects

Male C57BL/6 mice, aged 8 – 12 weeks old (Jackson Laboratory, Bar Harbor, ME) were initially housed in groups of four and maintained on a 12 hour light/dark cycle, food and water access was *ad libitum*. After intracranial cannulation surgery mice were singly housed for the remainder of the experiment. All training and testing occurred between the hours of 9:00 am and 7:00 pm. Housing and behavioral procedures were approved by the Temple University Institutional Animal Care and Use Committee.

Dose Selection

Two doses of nicotine hydrogen tartrate were selected, 0.09 ug/side and 0.18 ug/side (all doses reported in freebase). These doses were based on prior work demonstrating that both are sufficient to enhance forward trace fear

conditioning when infused into the DH and mPFC prior to training and testing (Raybuck & Gould, 2010).

Behavioral Procedure

Mice were conditioned in backwards trace conditioned safety as described in the previous section. However, due to limitations of drug infusion hardware and labor, mice were run in pairs. Additionally, single housed mice were removed from their home-cage and placed into a transfer cage and brought to the drug infusion room and then training/testing room. Transfer cages were labeled and only used for the same pair of mice for all training and testing sessions.

Drugs and Infusion

Nicotine hydrogen tartrate was dissolved in physiological saline, which was used as vehicle for control mice. Prior to infusion, mice were restrained and dummy cannula removed. Drug was directly infused using 22 gauge internal cannula (DH and VH) and 33 gauge internal cannula (PL) (Plastics One, Roanoke) attached to PE50 polyethylene tubing. Drug infusion rate for all three experiments was 0.5 ul/minute with a dosing volume of 0.5 ul per side. Infusions were controlled by microinfusion pump (KD Scientific, New Hope, PA) with 10 ul Hamilton syringe (Reno, NV). 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.

Surgical Procedure: Intracranial Cannulation

After being anesthetized with isoflurane (5% induction, 2.5% maintenance), mice were placed in a stereotaxic apparatus and implanted with a guide cannula. Bilateral guide cannulas (Plastics One) were placed for DH (A/P −1.7, M/L ±3.0, D/V −2.3 mm) and VH (A/P −2.8, M/L ±3.0, D/V −4.0 mm) (Raybuck & Gould, 2010). The rodent prefrontal cortex is anatomically different from that of the primate. However, the PL is a subregion of the mPFC that appears analogous to the primate dlPFC (Vertes, 2004). Thus, PL was the target for mPFC and coordinates were based on the stereotaxic location of PL (A/P +1.7, M/L ±0.5, D/V −2.5 mm). Mice were allowed to recover for at least 5 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 post-fixed in formalin for a minimum of 24 hours. Following fixation, brains were sectioned at 50 microns on cryostat and stained with cresyl violet. Confirmation of infusion sites was done using bright-field microscopy at 10X magnification to identify cannula tracks (Figure 9 and 10). Placements determined to fall outside of the target regions (23 mice total) were excluded from all analysis.

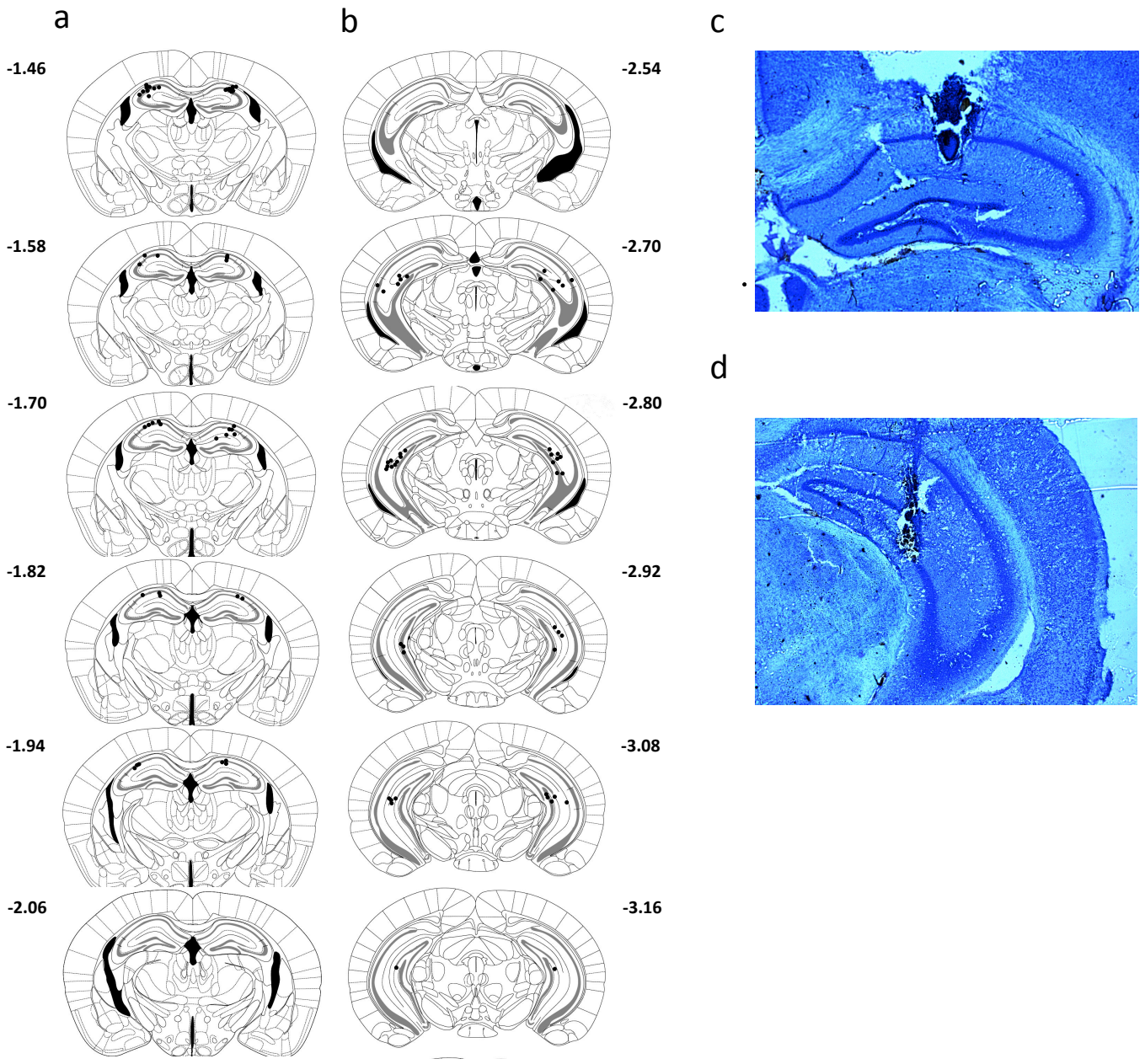

Figure 9: Cannula placement in hippocampus. Cannula tips location in relation to bregma in coronal sections (a) dorsal and (b) ventral hippocampus. Representative images of placements in dorsal (c) and ventral (d) hippocampus

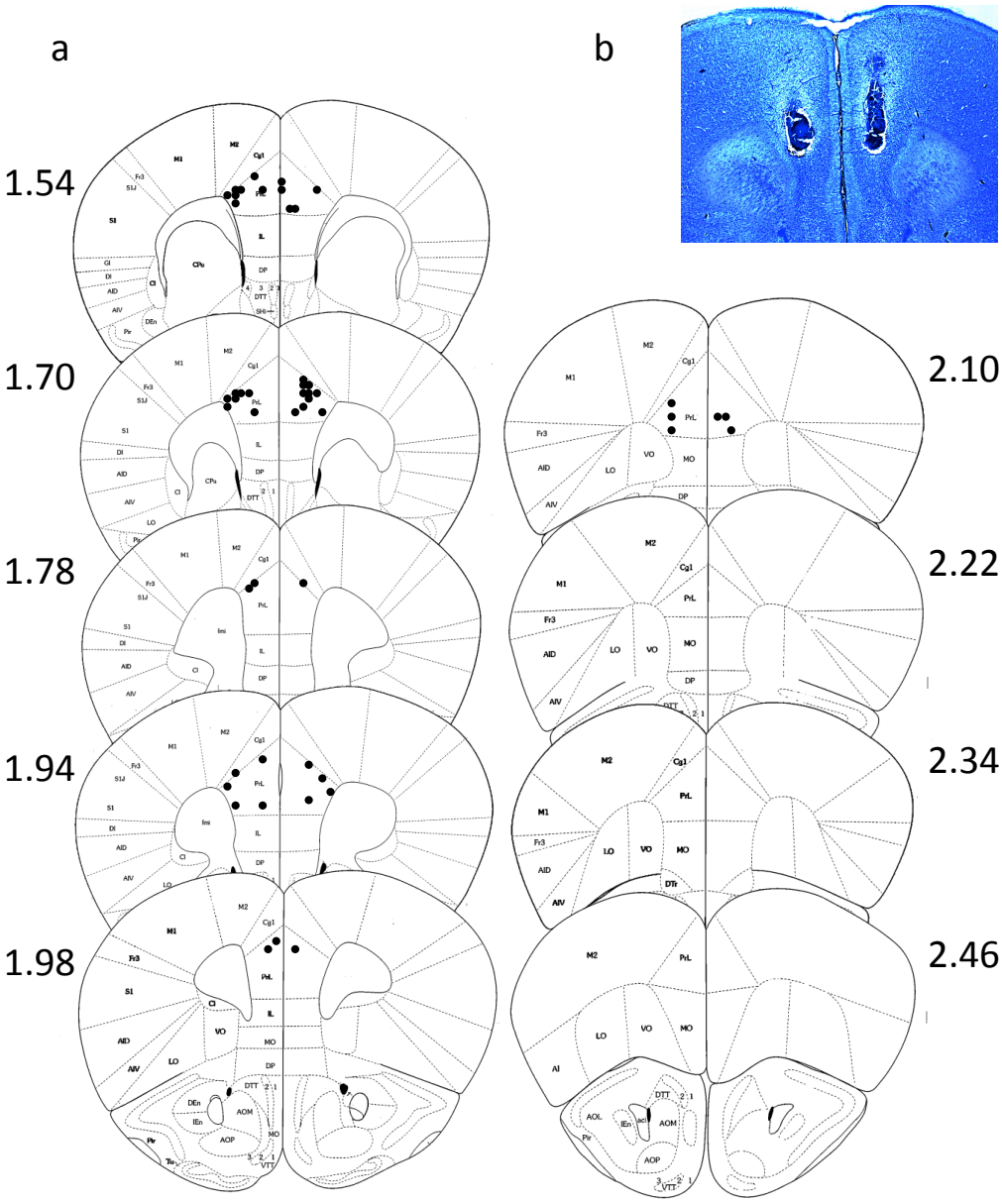

Figure 10: Cannula placement in prelimbic cortex (a) with representative image (b). Cannula tips location in relation to bregma in coronal sections

Data Analysis

For all three local administration studies, freezing data from Light and Light/Tone testing conditions was analyzed using a mixed-design ANOVAs. Planned comparison within-subjects *t*-tests were performed to assess changes in freezing between testing conditions. Post-hoc Tukey's HSD contrasts were used when necessary to compare between drug groups within a testing condition. Baseline freezing data, prior to presentation of any stimuli (Pre-CS), was analyzed with a one-way ANOVA. Results were considered significant at $p < 0.05$. All data are presented as means ± SEM. For all three experiments statistical analysis was performed using SPSS 16.0. One animal was removed from the 0.09 ug/side drug group for Pre-CS freezing 2 standard deviations higher than the rest of the group.

Results

Local administration of nicotine into DH dose dependently alters freezing at summation testing

Similar to the previously described systemic experiments, backward trace conditioned safety was assessed using a summation test. During summation testing mice were re-exposed to a novel context and presented with the Light-Danger or a compound Light/Tone-Safety. Therefore, formation of an inhibitory association with the backwards CS (Tone) was observed when mice showed decreased freezing during Light/Tone compound compared to presentation of the Light alone (Figure 11). A mixed-design ANOVA found a significant a significant interaction (Drug x Condition), $F_{(2,20)} = 3.79$, $p = 0.04$. Also a within subjects main effect of testing

condition was found (Danger vs. Safe), $F_{(1,20)} = 14.46$, $p = 0.001$, but no between subjects main effect was observed (Saline vs. 0.09 ug vs. 0.18 ug), $F_{(2,20)} = 3.145$, $p = 0.065$. 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 to Light alone (42.59%, SD = 10.39%), $t(8) = 3.221$, $p = 0.012$. Similarly, mice infused with 0.18 ug/side nicotine also froze significantly less during Light/Tone (29.37%, SD = 8.91%) compared to Light alone (41.27%, SD = 14.99%), $t(6) = 3.198$, $p = 0.019$. In contrast, mice administered 0.09 ug/side nicotine did not freeze differently during Light/Tone (50.00%, SD = 20.54%) compared to Light alone (50.00%, SD = 15.04%), $t(6) = 0$, $p = 1.00$. In addition, a one-way ANOVA revealed no significant variation in Pre-CS freezing among the different drug groups, $F_{(2,20)} = 0.237$, $p = 0.791$. Thus, no group differed in innate freezing or generalized freezing to the testing context.

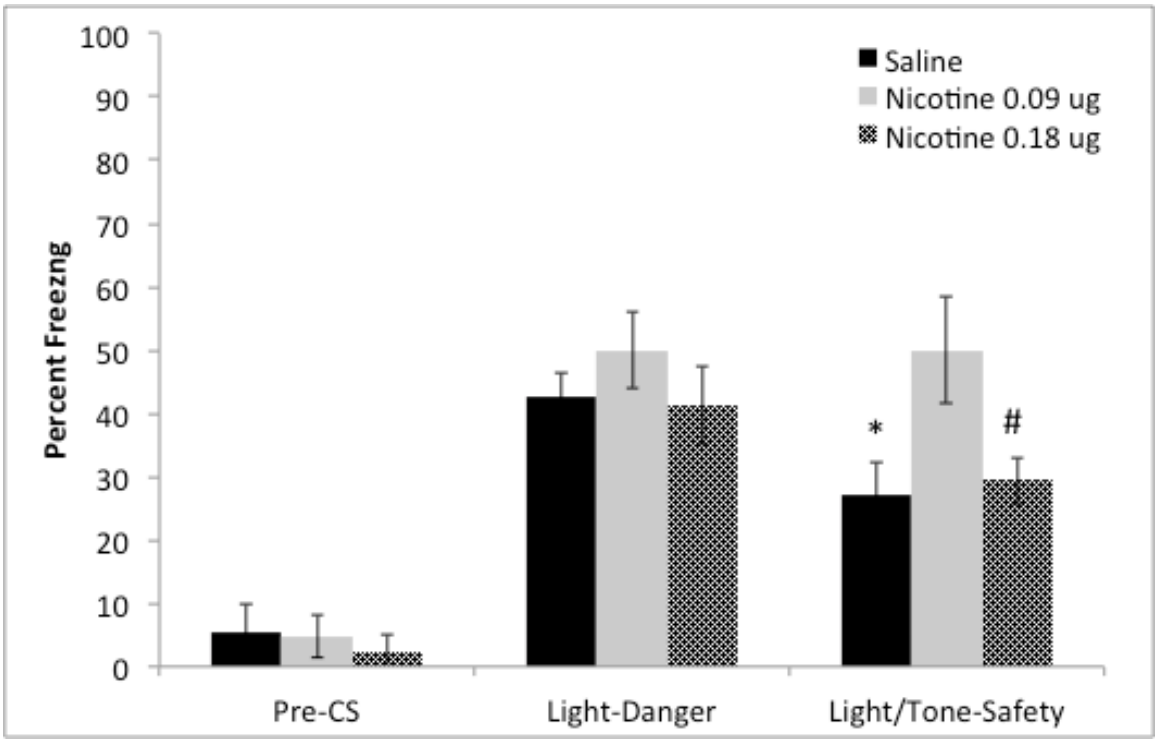

Figure 11: Nicotine in DH Dose-Dependently Alters Freezing to Light/Tone Compound. Mice administered saline and 0.18 ug/side nicotine showed significantly less freezing during presentation of compound light/tone compared to light alone. Mice treated with 0.09 ug/side nicotine had freezing levels similar during light and light/tone testing conditions. Error bars indicate SEM, (n = 7-9), (*) indicates significant planned comparison paired samples *t*-test (Light-Danger vs. Light/Tone-Safety) for saline group, $p < 0.05$. (#) Indicates significant planned comparison paired samples *t*-test (Light-Danger vs. Light/Tone-Safety) for 0.18 ug/side nicotine group, $p < 0.05$.

Local administration of nicotine into VH has no effect on freezing during summation testing

A mixed-design ANOVA found a significant within subjects main effect of testing condition (Danger vs. Safety), $F_{(1, 20)} = 43.024$, $p < 0.001$, indicating that mice responded differently to Light-Danger and Light/Tone-Safety presentations. No between subjects main effect of drug (Saline vs. 0.09 ug vs. 0.18 ug), $F_{(1, 20)} = 2.665$, $p = 0.094$ or interaction was observed, $F_{(2, 20)} = 0.036$, $p = 0.965$. The significant

between subjects main effect indicates that both saline and nicotine treatment groups learned safety. Specifically, mice administered saline froze less during compound Light/Tone (24.07%, SD = 16.43%) compared to Light alone (40.12%, SD = 13.26%). This effect of testing condition was also observed in mice infused with 0.09 nicotine, Light/Tone (14.29%, SD = 8.99%) compared to Light (31.75%, SD = 9.99%), and 0.18 nicotine, Light/Tone (26.19%, SD = 7.67%) compared to Light alone (43.65%, SD = 10.36%). Finally, assessment of Pre-CS freezing using a one-way ANOVA was not significant, $F_{(2,19)} = 1.080$, $p = 0.360$. Therefore, groups did not differ in innate freezing or show differences in generalized freezing to the testing context (Figure 12).

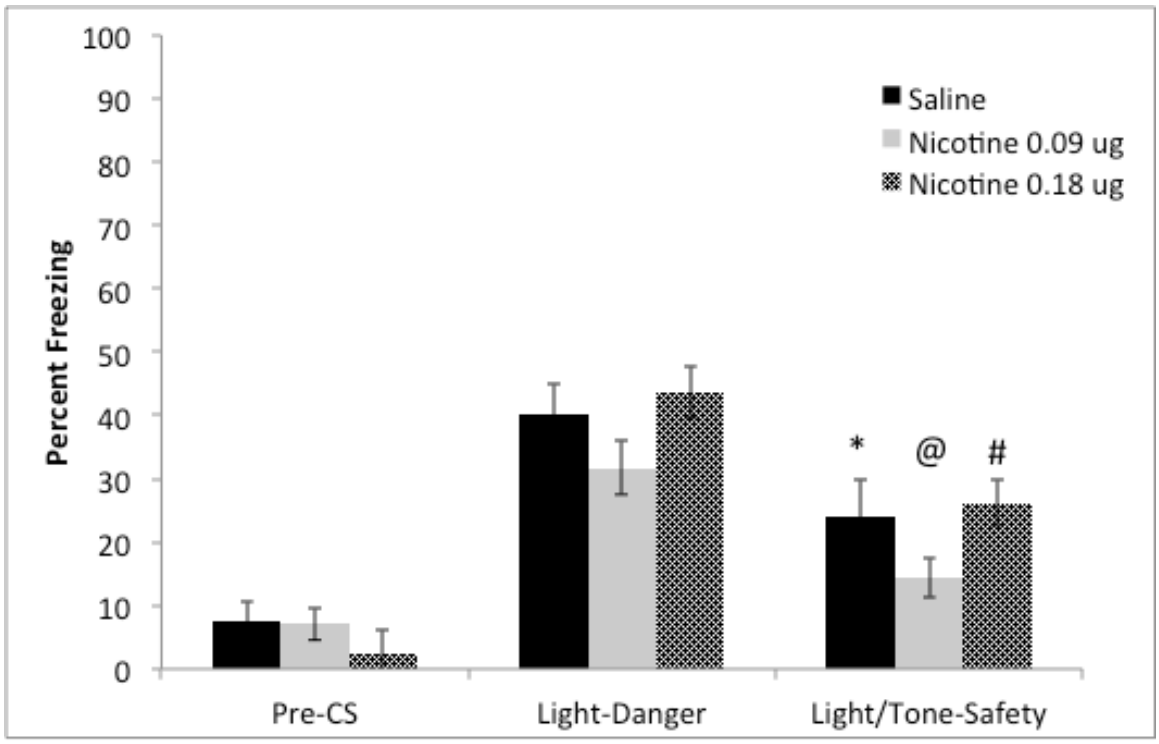

Figure 12: Nicotine in VH Has No Effect On Summation Testing, mice administered saline or 0.18 ug/side nicotine showed significantly less freezing during presentation of compound light/tone compared to light. Mice treated with 0.09 ug/side nicotine fail to show learned safety, with freezing levels similar between light and light/tone compound. Error bars indicate SEM, (n = 7-9), (*) indicates significant planned comparison paired samples *t*-test (Light-Danger vs. Light/Tone-Safety) for saline group, $p < 0.05$. (@) Indicates significant planned comparison paired samples *t*-test (Light-Danger vs. Light/Tone-Safety) for 0.09 ug/side nicotine group, $p < 0.05$. (#) Indicates significant planned comparison paired samples *t*-test (Light-Danger vs. Light/Tone-Safety) for 0.18 ug/side nicotine group, $p < 0.05$.

Local administration of nicotine into mPFC dose dependently alters freezing at summation testing

A mixed-design ANOVA revealed a significant interaction between drug and condition (Condition x Drug), $F_{(2,22)} = 9.482$, p = 0.002. In addition, a main effect of testing condition was seen (Danger vs Safety), $F_{(1,22)} = 35.762$, $p < 0.001$, but there was no significant between subjects main effect of drug (Saline vs. 0.09 ug vs. 0.18 ug), $F_{(2,22)} = 2.809$, $p = 0.082$. However, Therefore, the effect CS presentation was moderated by nicotine treatment. Moreover, a priori planned paired samples *t*-tests were performed. As expected, saline treatment was associated with significantly reduced freezing during Light/Tone trials (13.89%, SD = 8.91%) compared to Light alone (45.14%, SD = 13.09%), $t(7) = 9.00$, $p < 0.001$. Additionally, mice treated with 0.09 ug/side nicotine showed significantly reduced freezing during Light/Tone (37.04%, SD = 19.64%) compared to Light alone (53.09%, SD = 17.37%), $t(8) = 4.727$, $p = 0.001$. Similarly, treatment with 0.18 ug/side nicotine significantly reduced freezing during Light/Tone (22.22%, SD = 9.94%) compared to Tone alone (50.93%, SD = 11.34%), $t(5) = 5.270$, $p = 0.003$ (Figure 13).

Our a priori comparisons suggested all drug groups inhibited fear during Light/Tone-Safety. However visual inspection of the data, as well as a significant interaction from the mixed ANOVA suggested that drug groups froze differently during presentations of compound Light/Tone. To further examine this, post-hoc contrasts using Tukey's HSD were performed between Saline and both nicotine groups during Light/Tone-Safety, $F_{(2,22)} = 1.605$, $p = 0.018$. As a result, we found

that treatment with nicotine 0.09 ug/side resulted in freezing significantly higher (M=37.04%, SD = 19.64%) than saline (M=13.89%, SD = 8.91%), $p = 0.002$ during Light/Tone trials. No significant difference was found between Saline and 0.18 ug/side nicotine, $p = 0.290$. We considered that this difference could be the result of the control group freezing abnormally low. To assess this we also performed Tukey's contrasts within Light-Danger testing condition, $F_{(2,24)} = 6.74$, $p = 0.52$. No difference in freezing was observed between saline (M = 45.13%, SD = 13.09%) and nicotine 0.09 ug/side (M = 53.09%, SD 17.37%), $p = 0.515$ or saline and nicotine 0.18 ug/side (M = 52.78%, SD = 12.59%), $p = 0.559$. Finally, a one-way ANOVA found no significant differences in freezing during the Pre-CS period, $F_{(2,22)} = 1.605$, $p = 0.474$, indicating that mice did not differ in innate freezing or generalized freezing to the testing context.

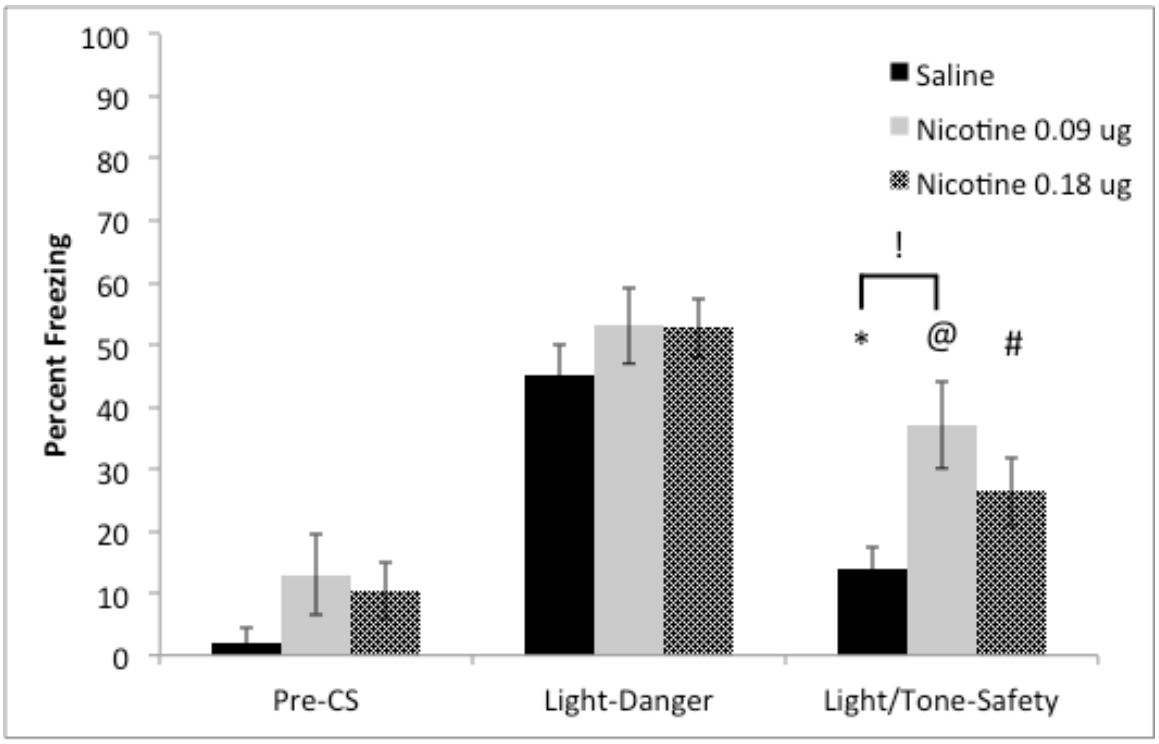

Figure 13: Nicotine in mPFC Dose-Dependently Alters Freezing to Light/Tone Compound, mice administered saline, 0.09 ug/side or 0.18 ug/side nicotine showed significantly less freezing during presentation of compound light/tone compared to light. Mice treated with 0.09 ug/side nicotine froze significantly more than saline treated mice during compound light/tone presentation. Error bars indicate SEM, (n = 8-9), (*) indicates significant planned comparison paired samples *t*-test (Light-Danger vs. Light/Tone-Safety) for saline group, $p < 0.05$. (@) Indicates significant planned comparison paired samples *t*-test (Light-Danger vs. Light/Tone-Safety) for 0.09 ug/side nicotine group, $p < 0.05$. (#) Indicates significant planned comparison paired samples *t*-test (Light-Danger vs. Light/Tone-Safety) for 0.18 ug/side nicotine group, $p < 0.05$. (!) Significant post-hoc Tukey's HSD between subjects comparison in reference to saline controls within Light/Tone-Safety, $p < 0.05$

Discussion

Summation testing showed that backward trace conditioned safety results in the formation of an inhibitory safety association with the backwards CS, however acute nicotine can disrupt this learning processes. Previously described data from Chapter 2 indicates that this disruption results from the formation of a maladaptive fear association with the backwards CS. For example, when nicotine was systemically administered during training and testing, we found that mice displayed fear in response to the backwards CS. Thus, it appears that the CS valence shifted from safety to fear upon nicotine administration. As a result, we interpreted our findings to suggest that nicotine acts indirectly on learned safety by facilitation a maladaptive US-CS fear. Importantly, learned safety is a form of conditioned inhibition and failure to form an inhibitory (CS-) association is sufficient to disrupt safety learning, however our results suggest that nicotine does not specifically block this inhibitory association, i.e., nicotine does not result in the absence of an association with the backwards CS. In contrast, nicotine facilitates formation of an excitatory (CS+) fear association with the backwards CS. Since excitatory/inhibitory behavioral output (freezing behavior) is one dimensional, the formation of the CS+ fear association intrinsically interferes with formation or expression of fear inhibition or safety. Again, this shift in valence appears to be contingent on temporal placement of US and CS, as mice administered acute nicotine systemically learned safety in the unpaired safety experiment. In sum, these data strongly indicate that the effects of acute nicotine on backwards trace

conditioned safety are the result of the formation of an association between the temporally discontiguous backwards paired US and CS.

In comparison to unpaired safety, during backwards conditioned safety the US and CS are reliably presented in close temporal proximity (20s trace interval). Moreover, forward trace conditioning, in which an excitatory (CS+) association is normally learned, depends upon cognitive mechanisms that allow learning temporally discontiguous associations. The hippocampus and mPFC have been shown to critically support trace learning and nicotine infused into these regions was previously shown to enhance trace fear conditioning (Raybuck & Gould, 2010). Therefore, nicotine, 0.09 and 0.18 ug/side, was locally administered bilaterally into the DH, VH, mPFC during training and testing of backwards trace conditioned safety to assess if these regions were involved in maladaptive learning. We found that nicotine infused into the DH and mPFC dose dependently enhanced formation of a US-CS fear memory. Thus, when nicotine was infused into the DH and mPFC, regions that have been implicated in formation and storage of trace memories, summation testing was disrupted. These behavioral data are consistent with our prior findings using acute nicotine, and suggest that the DH and mPFC may be critical for the effects of acute nicotine on backwards trace conditioned safety. In contrast, infusion into the VH did no alter behavioral responding to the backwards trace CS during summation testing. Therefore, while the VH has been shown to be important for fear learning and expression (Quinn et al., 2002), it does not appear critically involved in acute nicotine's effects on backwards trace conditioned safety. Importantly, the data presented here bolsters our initial assertion, that nicotine can

enhance learning of temporally discontiguous events irrespective of their temporal ordering. Likewise, this suggests that nicotine may make the DH and mPFC more permissive to establishing maladaptive associative fear memories.

Nicotine has differential effects in dorsal vs. ventral hippocampus on backwards trace conditioning

We found that infusion of nicotine into the DH resulted in enhanced formation of a fear US-CS association. As a result, this association interfered with learning the backwards trace CS as a safety cue observed during summation testing. In contrast, no effect of nicotine was observed when infused into the VH. As previously stated, the hippocampus is functionally and genetically divisible along the dorsal-ventral axis (Fanselow & Dong, 2010). Thus, nicotine's effects within the DH suggest that changes in cognitive process underlying associative learning are responsible for failure to adaptively respond to the safety cue during summation testing. In support, a study that found that backwards trace conditioning resulted in a mild fear association, but when the DH was lesioned, that association was abolished (Quinn et al., 2002). In addition, local administration of nicotine within the DH during training enhances trace fear conditioning (Raybuck & Gould, 2010). Thus, nicotine may act to enhance this initial association by altering processes within DH important for learning temporally discontiguous trace associations. Single unit recording within the dentate gyrus and CA1 regions showed learning related changes in firing in response to CS and US presentations after trace fear conditioning. Moreover, nicotine has been shown to alter neural excitability and lower the threshold for LTP in both of these regions (Ji, Lape, & Dani, 2001; Welsby,

2009). Therefore, our results suggest that nicotine might alter learning-related plasticity within DH, facilitating a maladaptive fear association. Finally, due to the fact that nicotine was administered during training and testing we can not be certain that the effects of nicotine are not due to changes in memory recall. However, previous work has shown that when nicotine was directly infused into the DH, the effects on long-term memory are dependent on pre-training administration, but not pre-testing (Kenney, Raybuck, & Gould, 2012). Moreover, manipulations of the DH do not generally cause intrinsic changes in fear expression and the DH does not make direct connections to the amygdala (Corcoran & Maren, 2001; Pitkänen et al., 2000). Therefore, the effects of nicotine in the DH on freezing behavior are more likely the result in changes in maladaptive memory acquisition and consolidation.

In contrast to DH infusions, VH administration had no effect on backwards conditioned safety. This null effect supports the interpretation that nicotine's effects on backwards trace conditioning are mediated by enhanced maladaptive associative learning. For example, previous of studies have found that infusion of nicotine into the VH actually disrupts associative learning (Kenney et al., 2012; Raybuck & Gould, 2010). Therefore infusion of nicotine into the VH likely has no effect here because under normal conditions backwards trace conditioning results in little or no CS-US association. That is to say, because learning safety requires that an association between the US and CS *not* be formed, there is no association to disrupt. Therefore, our data does not appear in conflict with prior work showing that VH nicotine administration causes deficits in hippocampus-dependent

learning. In addition, we hypothesized that VH nicotine administration might disrupt learned safety as the VH is involved in emotional processing (Fanselow & Dong, 2010; Stephen Maren, 2008). However, our results suggests that the VH may not be a critical for nicotine's effects on backwards trace conditioning. This conclusion is also supported by work that found inactivation of VH did not prevent discrimination of dangerous and safe cues (Chen, Foilb, & Christianson, 2016). Thus, while VH plays an important role in fear expression, as observed in other fear conditioning and fear extinction work (Kjelstrup et al., 2002; Sierra-Mercado, Padilla-Coreano, & Quirk, 2011), our data supports a view that it may not be similarly important for learned safety. In sum, these data suggest that the VH is not sufficient to mediate the effect of acute nicotine on backwards conditioned safety.

The mechanism by which nicotine modulates DH function to alter learning of the backwards trace CS is not known, but is likely mediated by nAChRs. In support, nicotine-associated changes in cognition are dependent on nAChRs and alterations in cholinergic signaling can modulate cognition (Davis & Gould, 2006, 2007; Woodruff-Pak, 2003). Activation of nAChRs can result in influx of calcium, leading to modulation of intracellular cell signaling cascades (Brunzell, Russell, & Picciotto, 2003; Nakayama, Numakawa, Ikeuchi, & Hatanaka, 2001). Further, nicotine has been shown to interact with molecular mechanisms of long-term memory within the hippocampus, including shifting the expression of CREB and altering the expression of hippocampal learning-related kinases (Gould, Wilkinson, Yildirim, Poole, et al., 2014; Kenney, Poole, Adoff, Logue, & Gould, 2012). Therefore, nicotine can alter intracellular mechanisms of learning via nAChRs to facilitate

changes in associative learning. In addition, nicotine can lower the threshold of hippocampal LTP induction (Ji et al., 2001) and cholinergic signaling via nAChRs can modulate timing-dependent plasticity in CA1 (Gu, Lamb, & Yakel, 2012). Thus, nicotine might facilitate potentiation of synapses under conditions in which LTP is disadvantageous, such as during inhibitory learning, i.e., learned safety. Interestingly, nicotine has also been shown to alter hippocampal LTP in a D1 receptor-dependent manner and activation of D1 receptors can shift the window of hippocampal spike-timing dependent plasticity (STDP), increasing the likelihood of LTP (Tang & Dani, 2009; Yang & Dani, 2014). Considering that STDP has been proposed as a mechanism mediating backwards conditioning (Gerber et al., 2014), such alterations could be involved in nicotine's effect on backwards trace conditioned safety described here. For example, nicotine might widen the temporal window within which LTP occurs, facilitating the strengthening of synaptic connections that underlie maladaptive associative learning. Such changes in plasticity could be problematic and normal homeostatic mechanisms likely regulate neural plasticity to maximize adaptive function (Franklin, Fickbohm, & Willard, 1992). That is to say, changes in molecular signaling and synaptic efficiency that are more permissive to forming associations could enhance learning under some conditions, but could also result in maladaptive learning under alternative conditions. Additionally, nicotine might shift molecular and physiological mechanisms within the hippocampus into a state that is biased towards the formation of fear associations that compete with more adaptive safety associations.

The role of the hippocampus in safety learning is still not clear, however the limited work available suggests that conditioned inhibition of fear can occur independent of the hippocampus (Chen et al., 2016; Heldt et al., 2002). Furthermore, considering that systemic nicotine did not disrupt unpaired learned safety, the effect of DH nicotine on backwards trace conditioning suggests that acute nicotine alters trace learning mediated by DH processes, but not more fundamental aspects of learned safety. In support, work has shown that feature-negative safety discrimination can occur in the absence of hippocampus (Heldt et al., 2002; Kazama, Heuer, Davis, & Bachevalier, 2012). Thus, nicotine may facilitate maladaptive DH processing normally inhibited or absent during backwards trace conditioning. However, it should be noted that the explicitly unpaired safety procedure reported here was previously shown to alter hippocampal neurogenesis and that ablation of neurogenesis was able to retard safety learning (Pollak et al., 2008). Therefore, an important methodological consideration is that differences in safety conditioning paradigms could result in recruitment of different brain regions and cognitive mechanisms. In this respect, fear conditioning may provide a useful analogy, as it is well known that fear conditioning circuitry is dramatically altered depending on the nature of the conditioning paradigm. For example, some forms of fear learning critically depend on the hippocampus, i.e., contextual and trace, while other forms of fear learning have been shown to be hippocampus-independent (Bast, Zhang, & Feldon, 2003; Chowdhury et al., 2005; Fendt et al., 2005; Phillips & LeDoux, 1994; Quinn et al., 2005). Thus, as learned safety is investigated further, different training paradigms may become associated with specific brain regions

required for specific forms of safety learning. However, learned safety likely has a default circuit that that is fundamental, analogous to our current understanding of the thalamic and amygdala circuitry critical for all forms of fear learning. Currently, work suggests the sensory insula may play a role in this circuit (Christianson et al., 2008). In sum, it is not clear under which conditions of learned safety the hippocampus plays a critical role, however we find that nicotine within the DH can interfere with backwards trace conditioned safety.

mPFC nicotine administration enhances backwards trace US-CS association

Evidence suggests that mPFC supports trace conditioning via working memory processes (Gilmartin, Kwapis, et al., 2013; Gilmartin, Miyawaki, et al., 2013), therefore we infused nicotine into the mPFC to assess effects on backwards trace conditioned safety. We found that summation testing of a backwards trace CS was intact in nicotine treated mice, i.e., mice froze less during Light/Tone-Safety compared to Light-Danger. However, freezing was significantly increased when nicotine (0.09 ug/side) was infused into the mPFC. Data from control animals in this study showed lower freezing during Light/Tone-Safety compared to other studies. However, we do not believe that this is due to abnormal behavior as there was no difference in freezing behavior during Pre-CS or Light presentations. This finding indicates that nicotine within the mPFC enhanced backwards trace US-CS learning. Interestingly, prior work found that learned safety was not disrupted by lesions of the mPFC (Christianson et al., 2008; Gewirtz, Falls, & Davis, 1997). Therefore, our mPFC data further supports our hypothesis that acute nicotine acts on trace associative learning, but not directly on mechanisms mediating learned

safety, and nicotine within the mPFC exerts specific effects on associative learning of temporally discontiguous stimuli.

Nicotine may act on intrinsic properties of the mPFC including modulation of neural activity during the backwards trace interval during training. For example, cells within the PL were found to maintain sustained firing during the trace interval during forward trace fear conditioning (Gilmartin & McEchron, 2005) and inhibition of PL neurons during the trace interval disrupted trace fear learning (Gilmartin, Miyawaki, et al., 2013). Therefore, during backwards trace conditioning, nicotine could possibly increase or modulate mPFC neural activity during the trace interval. It is well established that the mPFC plays an important role in working memory (Ragozzino et al., 2002) and nicotine can enhance working memory in rats and humans (Levin & Torry, 1996; Provost & Woodward, 1991). If nicotine within the mPFC enhances working memory during backwards trace conditioning, this could stabilize or strengthen a representation of the US across the trace interval. Interestingly, some theoretical accounts of backwards conditioned safety suggest that the organism forms an association between relief, from the removal of the aversive US, and the CS (Gerber et al., 2014; Mohammadi et al., 2014). Such an account of backwards conditioning of safety would require that the US *not* be highly salient at the time the CS is presented. However, if nicotine within the mPFC enhances the working memory representation of the US, the US may still be highly salient during presentation of the CS during backwards trace conditioning, facilitating the formation of a maladaptive backwards US-CS association. Finally, while this study was not designed to determine if nicotine's effects within the mPFC

are the result of changes in acquisition or recall, prior work found that pre-testing infusion of nicotine into the mPFC had no effect on recall during trace fear conditioning. Therefore the most parsimonious explanation is that nicotine modulates mPFC substrates involved in working memory processes during acquisition of backwards trace.

The effects of nicotine within the mPFC on backwards trace conditioned safety are likely mediated by nAChRs. nAChRs are expressed within the mPFC (Eppolito, Bachus, McDonald, Meador-Woodruff, & Smith, 2010) and are involved in cognitive processes including attention (Counotte et al., 2011; Guillem et al., 2011) and working memory (Levin & Torry, 1996; Levin & Rose, 1991). Additionally, nAChR activation is associated with release of neurotransmitters important for modulating attention/working memory processes, including glutamate, dopamine, acetylcholine, and noradrenalin (Parikh, Man, Decker, & Sarter, 2008). Specifically, activation of mPFC nAChRs by nicotine has been shown to increase amplitude of cholinergic transients and induce glutamate release (Parikh et al., 2008). Changes in glutamate release may be important in relation to sustained neural firing within prefrontal cortex shown to be recruited for trace conditioning (Gilmartin, Miyawaki, et al., 2013). Further, blockade of nAChRs in the PL disrupted working memory in a delay match-to-sample task (Granon, Poucet, Thinus-Blanc, Changeux, & Vidal, 1995). Therefore, nicotine may act on nAChRs within the mPFC altering working memory processes during the backwards trace interval, facilitating maladaptive associative learning. In addition, the mPFC is highly innervated by dopamine neurons and it is well established that sustained

cortical release of dopamine is critical for working memory processes (Brozoski, Brown, Rosvold, & Goldman, 1979). Indeed, infusion of nicotine into prefrontal cortex has been shown to result in increased levels of extracellular dopamine (Shearman, Rossi, Sershen, Hashim, & Lajtha, 2005). Thus, changes in synaptic concentrations of dopamine could modulate working memory processes during trace conditioning. In support, administration of amphetamine and methylphenidate enhanced trace fear conditioning (Horsley & Cassaday, 2007; Norman & Cassaday, 2003), which suggests that nicotine induced dopamine release within the mPFC could play a role in facilitating a maladaptive US-CS association during backwards conditioning.

Nicotine dose dependently alters learning backwards trace CS as a safety cue

Local administration of nicotine into the DH and mPFC dose dependently disrupted backwards trace conditioned safety, with the highest dose of nicotine (18 ug/side) showing no change in learning. This dose specific effect on backwards learned safety suggests that nicotine is acting on substrates of cognition. Indeed, disruption of function generally follows an asymptotic dose response pattern with negative slope. For example, blockade of NMDARs results in decreased learning as dosage increases (Gould, McCarthy, & Keith, 2002). In contrast, pharmacological modulation or enhancement of function is highly sensitive to endogenous and non-specific pharmacological effects and will often present as a non-monotonic dose response. For example, cocaine-associated changes in motivated behavior fail to occur at low and high doses (Caine et al., 2002). Moreover, our results 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, enhancement of hippocampus-dependent learning by acute nicotine shows a similar dose response curve (Gould & Higgins, 2003). Moreover, enhancement of forward trace fear conditioning by DH and mPFC nicotine infusions also showed a an inverted U-shaped dose response (Raybuck & Gould, 2010). Therefore, our results suggest that nicotine within the DH and PL has decreasing effects on associative learning during backwards trace conditioning at higher doses.

The range of doses at which nicotine exerts changes on cognition may be related to dynamics of nAChRs activation and desensitization (Picciotto, 2003). Thus, the effects of nicotine are likely subject to a balance between activation and desensitization of nAChRs (Fenster, Rains, Noerager, Quick, & Lester, 1997; Quick & Lester, 2002) with higher concentrations of nAChR agonist associated with increased desensitization (Katz & Thesleff, 1957) and repeated administration resulting receptor upregulation (Fenster et al., 1997). In addition, endogenous acetylcholine is released during learning may also modulate nAChR desensitization (Hasselmo, 2006). Therefore, lower doses of nicotine may activate nAChRs, while higher nicotine concentrations may lead to desensitization resulting in a decrease in nicotine's ability to activate nAChRs. In addition, nAChRs are a heterogeneous group of receptors with differing affinities for nicotine and differences in regional expression (Gould, 2006). Moreover, activation nAChRs can engage competing mechanisms, such as the release of both glutamate and GABA within hippocampus (Radcliffe, Fisher, Gray, & Dani, 1999), as well modulate the threshold for

hippocampal LTP and long-term depression (Ge & Dani, 2005). Thus, phenomenological cognitive effects of nicotine likely require the interaction of overlapping cell populations and receptor subtypes.

Local administration data described here indicates that the effects of nicotine in the DH and mPFC on backwards trace conditioning are similar to findings from forward trace fear conditioning. However, there may be some subtle differences between the effects of nicotine on backwards and forward trace conditioning. For example, bilateral local administration of nicotine (0.18 ug/side) within the DH and mPFC enhanced forward trace fear conditioning. In contrast, we observed no difference in backwards trace conditioning at this higher dose. Most likely, differences in dose response are the result of methodological differences between studies. In support, Raybuck and Gould (2010) found that sensitivity to the effects of DH nicotine infusion on contextual learning were sensitive to trials number. For example, mice trained with 2 trials showed enhanced contextual learning with 35 ug/side, while training with 5 trials resulted in enhancement at 0.09 ug/side. These data suggest that sensitivity to the cognitive effects of acute nicotine may increas as conditioning trials increase in number. Considering that the backwards conditioning paradigm uses a total of 15 trials, compared to 5 trials in trace fear conditioning, a leftward shift in dose response may then be expected. Importantly, it should be noted that the effective dose, 0.09 ug/side, used in backwards trace conditioned safety did overlap with an effective dose in forward trace fear conditioning. Thus, the local effects of nicotine within the DH and mPFC

in the backwards trace conditioning paradigm align well with prior work using forward trace fear conditioning.

CHAPTER 4

CONCLUSION

The experiments described in Chapters 2 and 3 are the first to assess the effects of nicotine on learned safety. In Chapter 2 we investigated the effects of systemically administered acute nicotine in two paradigms of learned safety, unpaired and backwards trace, and found that nicotine specifically altered backwards trace conditioned safety. Specifically, when presented in compound with a danger-associated cue, the backwards trace CS did not act as a conditioned inhibitor of fear or safety cue. Additionally, when mice received chronic nicotine, this effect in backwards trace conditioned safety was not observed, suggesting that tolerance develops to the effects of nicotine with continued exposure. These data provide evidence that nicotine does not intrinsically block conditioned inhibition of fear by a discrete safety cue. Rather, acute nicotine can facilitate enhanced fear association with a backwards trace CS. In sum, these data show that acute nicotine can result in maladaptive learning of the US-CS association in backwards trace conditioning, which results in a failure to behaviorally respond to the backwards CS as a safety cue.

In follow up to the experiments in Chapter 2, studies in Chapter 3 consisted of behavioral pharmacology experiments to identify brain regions that may be critical for acute nicotine-associated effects on backwards trace conditioned safety. We selected the hippocampus and mPFC as target regions because prior work has indicated that these regions mediate learning of trace associations (Raybuck &

Lattal, 2014). Additionally, a prior study found that nicotine infused into the DH and mPFC enhanced forward trace fear conditioning (Raybuck & Gould, 2010). In support, we found that nicotine infused into the DH and mPFC could disrupt learning the backwards trace CS as a safety cue. These results support an interpretation that nicotine acts on backwards trace conditioned safety by facilitating a maladaptive US-CS trace association.

The studies described here were all performed in male mice and therefore the scope of the work is limited in this respect. Human literature indicates that women develop PTSD at twice the rate of men (Inslicht et al., 2013) and that there are sex-specific difference in genetic risk (Ressler et al., 2011). As a result, preclinical work has sought to investigate a biologically based mechanism mediating sex-related differences in PTSD risk, including differences in stress induced neural-adaptations (Bangasser & Valentino, 2012; Valentino, Bangasser, & Van Bockstaele, 2013). Therefore, nicotine-associated changes in learned safety might be more pronounced or present differently in female subjects compared to males. While there is a paucity of work investigating sex specific differences in learned safety, one study did find that trauma exposed girls (age 8-13) showed poorer discrimination between safe and danger cues compared to boys (Gamwell et al., 2015). Therefore, future studies are needed to investigate sex specific differences in learning safety as it relates to PTSD. It should also be noted that prior work suggests nicotine can modulate hippocampus-dependent fear learning in males and females similarly (Davis & Gould, 2007; Leach et al., 2015). Therefore, it is unclear how nicotine would alter safety learning in female subject and future

studies aimed at explicitly investigating sex differences in the effects of nicotine on learned safety are necessary.

Nicotine administration strategies employed in all experiments follow well established pharmacological modeling of acute and chronic exposure (Davis et al., 2007; Kenney et al., 2011; Kutlu et al., 2014; Portugal, Wilkinson, Kenney, et al., 2012); however, these pharmacological models are limited in their applicability to human smokers. For example, similar to previous preclinical studies using the same dose and route-of-administration, we found no effect of chronic nicotine on associative learning (Davis & Gould, 2009; Gould, Wilkinson, Yildirim, Blendy, et al., 2014; Raybuck & Gould, 2009). In contrast, clinical chronic smoking populations have been found to present with cognitive deficits (Durazzo, Meyerhoff, & Nixon, 2010). One reason for this disparity may be slight differences in the delivery of nicotine in our chronic model and that in smokers. For example, in our chronic model nicotine is delivered 24 hours a day, while tobacco users would likely not be exposed to nicotine non-cyclically. Additionally, while our nicotine studies are relevant to tobacco product users, it should be noted that tobacco smoke is a complex mixture of over 5,000 different chemicals (Talhout et al., 2011). Thus, while nicotine is a critical factor in understanding the cognitive effects of tobacco use, our results are limited to understanding the relationship between nicotine and learned safety. Further work explicitly investigating tobacco smoke and learned safety is still needed to provide a more comprehensive understanding of the relationship between smoking, learned safety, and PTSD.

Altogether these studies provide insight and extend our understanding of the relationship between nicotine and cognition. Most interestingly, we show here that acute nicotine-associated effects on cognition, which may facilitate some learning, may come at the cost of other forms of learning. Specifically, we show here that acute nicotine-associated enhancement of a backwards trace danger associations, may form at the cost of competing safety associations. It has been repeatedly observed that nicotine can enhance hippocampus-dependent learning. However, the findings described here suggest that terms such as "enhancement" and "disruption" may be too binary to fully capture the effects of acute nicotine. Other work supports this notion and suggests that nicotine's complex effects on cognition are not limited to emotional learning. For example, acute nicotine resulted in enhance hippocampus-dependent spatial object recognition, but induced deficits in novel object recognition (Kenney et al., 2011). Considering that novel object learning does not depend critically on the hippocampus, this data suggest that nicotine may enhance some types of hippocampus-dependent associative learning, but with a cost to non-hippocampal forms of learning. While we did not directly investigate the role of the hippocampus in forming backwards trace safety associations, our finding suggests also that nicotine biases learning towards facilitation of the US-CS association over the safety association. Interestingly, this finding also suggests that nicotine engages processes that are either under inhibition or otherwise disengaged during backwards trace conditioning. One possible mechanism worth further study is nicotine's effects on inhibitory circuits. For example, nicotine has been shown to disinhibit local circuits

in the dentate gyrus and to induce hippocampal theta (Ji & Dani, 2000; Lu, Li, Li, & Henderson, 2013). Theta oscillations are highly regulated by inhibitory neurons and may play a role in pattern separation, which is critical for correctly discriminating between inputs (Buzsáki, 2002; Myers & Scharfman, 2011). This discrimination might be critical for backwards trace conditioned safety considering that two different CS's are presented in close proximity to the US. Therefore, an interesting hypothesis is that nicotine modulates hippocampal theta allowing for more robust, but less temporally specific associations to be formed.

This study shows for the first time that nicotine can alter a learning process that appears highly adaptive and is dysfunctional in individuals with PTSD. As stated previously, the behavioral phenomenon of inhibitory backwards conditioning is conserved across a number of species from insects to mammals. Moreover, this conservation suggests backwards conditioning may reveal an important learning rule or causal inference algorithm. Indeed, temporal ordering is a critical component for inferring cause and effect. That is, if event A occurs in time before event B then event A cannot be caused by event B. Furthermore, similar learning rules based on temporal ordering can be observed at the neural physiological level in the example of spike-time dependent plasticity. During spike-time dependent plasticity, if an input spike of a neuron precedes an output spike then LTP is more likely to be established. In contrast, if the input spike occurs after the output spike, induction of LTD is more likely. Thus, evolution may favor usage of temporal information in computing inhibitory behavioral/neural outputs and nicotine might cause deleterious effects by biasing temporal learning rules.

Additionally, our results also have implication for understanding the relationship between nicotine use and PTSD. For example, here we found that altered cognition resulted in a change of adaptive learning and behavioral response to safety cues. Indeed, there are likely multiple ways in which nicotine use may alter cognition to facilitate PTSD symptomology. However, we show that under some conditions nicotine can alter learning of cues that indicate safety. Our data suggests nicotine may facilitate formation of maladaptive danger associations with cues that actually predict the absence of threat. Therefore, nicotine induced changes in higher order cognition, which do not act directly on emotional processing, may potentiate PTSD symptomology.

REFERENCES CITED

Acheson, D. T., Gresack, J. E., & Risbrough, V. B. (2012). Hippocampal dysfunction effects on context memory: Possible etiology for posttraumatic stress disorder. *Neuropharmacology*, *62*(2), 674–685. http://doi.org/10.1016/j.neuropharm.2011.04.029

Amitai, N., & Markou, A. (2009). Chronic nicotine improves cognitive performance in a test of attention but does not attenuate cognitive disruption induced by repeated phencyclidine administration. *Psychopharmacology*, *202*(1-3), 275–286. http://doi.org/10.1007/s00213-008-1246-0

Astur, R. S., St. Germain, S. A., Tolin, D., Ford, J., Russell, D., & Stevens, M. (2006). Hippocampus Function Predicts Severity of Post-Traumatic Stress Disorder. *CyberPsychology & Behavior*, *9*(2), 234–240. http://doi.org/10.1089/cpb.2006.9.234

Bangasser, D. A., & Valentino, R. J. (2012). Sex Differences in Molecular and Cellular Substrates of Stress. *Cellular and Molecular Neurobiology*, *32*(5), 709–723. http://doi.org/10.1007/s10571-012-9824-4

Bangasser, D. A., Waxler, D. E., Santollo, J., & Shors, T. J. (2006). Trace Conditioning and the Hippocampus: The Importance of Contiguity. *The Journal of Neuroscience*, *26*(34), 8702–8706. http://doi.org/10.1523/JNEUROSCI.1742-06.2006

Bast, T., Zhang, W.-N., & Feldon, J. (2003). Dorsal hippocampus and classical fear conditioning to tone and context in rats: Effects of local NMDA-receptor blockade and stimulation. *Hippocampus*, *13*(6), 657–675. http://doi.org/10.1002/hipo.10115

Bremner, J. D., Southwick, S. M., Darnell, A., & Charney, D. S. (1996). Chronic PTSD in Vietnam Combat Veterans: Course of Illness and Substance Abuse. *American Journal of Psychiatry*, *153*(3), 369.

Breslau N, Kessler RC, Chilcoat HD, Schultz LR, Davis GC, & Andreski P. (1998). Trauma and posttraumatic stress disorder in the community: The 1996 detroit area survey of trauma. *Archives of General Psychiatry*, *55*(7), 626–632. http://doi.org/10.1001/archpsyc.55.7.626

Brozoski, T. J., Brown, R. M., Rosvold, H. E., & Goldman, P. S. (1979). Cognitive Deficit Caused by Regional Depletion of Dopamine in Prefrontal Cortex of Rhesus Monkey. *Science*, *205*(4409), 929–932.

Brunzell, D. H., Russell, D. S., & Picciotto, M. R. (2003). In vivo nicotine treatment regulates mesocorticolimbic CREB and ERK signaling in C57Bl/6J mice. *Journal of Neurochemistry*, *84*(6), 1431–1441. http://doi.org/10.1046/j.1471-4159.2003.01640.x

Bryant, R. A., Felmingham, K., Kemp, A., Das, P., Hughes, G., Peduto, A., & Williams, L. (2008). Amygdala and ventral anterior cingulate activation predicts treatment response to cognitive behaviour therapy for post-traumatic stress disorder. *Psychological Medicine*, *38*(04), 555–561. http://doi.org/10.1017/S0033291707002231

Buzsáki, G. (2002). Theta Oscillations in the Hippocampus. *Neuron*, *33*(3), 325–340. http://doi.org/10.1016/S0896-6273(02)00586-X

Caine, S. B., Negus, S. S., Mello, N. K., Patel, S., Bristow, L., Kulagowski, J., … Borrelli, E. (2002). Role of dopamine D2-like receptors in cocaine self-administration: studies with D2 receptor mutant mice and novel D2 receptor antagonists. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *22*(7), 2977–2988. http://doi.org/20026264

Carew, T. J., & Wehner, J. M. (1999). Nicotine enhancement of contextual fear conditioning. *Behavioural Brain Research*, *102*(1–2), 31–39. http://doi.org/10.1016/S0166-4328(98)00157-0

Carter, R. M., Hofstötter, C., Tsuchiya, N., & Koch, C. (2003). Working memory and fear conditioning. *Proceedings of the National Academy of Sciences*, *100*(3), 1399–1404. http://doi.org/10.1073/pnas.0334049100

Chen, V. M., Foilb, A. R., & Christianson, J. P. (2016). Inactivation of ventral hippocampus interfered with cued-fear acquisition but did not influence later recall or discrimination. *Behavioural Brain Research*, *296*, 249–253. http://doi.org/10.1016/j.bbr.2015.09.008

Chowdhury, N., Quinn, J. J., & Fanselow, M. S. (2005). Dorsal Hippocampus Involvement in Trace Fear Conditioning With Long, but Not Short, Trace Intervals in Mice. *Behavioral Neuroscience October 2005*, *119*(5), 1396–1402.

Christianson, J. P., Benison, A. M., Jennings, J., Sandsmark, E. K., Amat, J., Kaufman, R. D., … Maier, S. F. (2008). The Sensory Insular Cortex Mediates the Stress-Buffering Effects of Safety Signals But Not Behavioral Control. *The Journal of Neuroscience*, *28*(50), 13703–13711. http://doi.org/10.1523/JNEUROSCI.4270-08.2008

Christianson, J. P., Fernando, A. B. P., Kazama, A. M., Jovanovic, T., Ostroff, L. E., & Sangha, S. (2012). Inhibition of Fear by Learned Safety Signals: A Mini-Symposium Review. *The Journal of Neuroscience*, *32*(41), 14118–14124. http://doi.org/10.1523/JNEUROSCI.3340-12.2012

Christianson, J. P., Jennings, J. H., Ragole, T., Flyer, J. G. N., Benison, A. M., Barth, D. S., … Maier, S. F. (2011). Safety Signals Mitigate the Consequences of Uncontrollable Stress Via a Circuit Involving the Sensory Insular Cortex and Bed Nucleus of the Stria Terminalis. *Biological Psychiatry*, *70*(5), 458–464. http://doi.org/10.1016/j.biopsych.2011.04.004

Connor, D. A., & Gould, T. J. (2016). The role of working memory and declarative memory in trace conditioning. Submitted to Neurobiology of Learning and Memory

Corcoran, K. A., & Maren, S. (2001). Hippocampal Inactivation Disrupts Contextual Retrieval of Fear Memory after Extinction. *The Journal of Neuroscience*, *21*(5), 1720–1726.

Cougle, J. R., Zvolensky, M. J., Fitch, K. E., & Sachs-Ericsson, N. (2010). The role of comorbidity in explaining the associations between anxiety disorders and smoking. *Nicotine & Tobacco Research*, *12*(4), 355–364. http://doi.org/10.1093/ntr/ntq006

Counotte, D. S., Goriounova, N. A., Li, K. W., Loos, M., Schors, R. C. van der, Schetters, D., … Spijker, S. (2011). Lasting synaptic changes underlie attention deficits caused by nicotine exposure during adolescence. *Nature Neuroscience*, *14*(4), 417–419. http://doi.org/10.1038/nn.2770

Cunningham, C. L. (1981). Association between the elements of a bivalent compound stimulus. *Journal of Experimental Psychology. Animal Behavior Processes*, *7*(4), 425–436.

Davis, J. A., & Gould, T. J. (2006). The effects of DHBE and MLA on nicotine-induced enhancement of contextual fear conditioning in C57BL/6 mice. *Psychopharmacology*, *184*(3-4), 345–352. http://doi.org/10.1007/s00213-005-0047-y

Davis, J. A., & Gould, T. J. (2007). β2 subunit-containing nicotinic receptors mediate the enhancing effect of nicotine on trace cued fear conditioning in C57BL/6 mice. *Psychopharmacology*, *190*(3), 343–352. http://doi.org/10.1007/s00213-006-0624-8

Davis, J. A., & Gould, T. J. (2009). Hippocampal nAChRs mediate nicotine withdrawal-related learning deficits. *European Neuropsychopharmacology*, *19*(8), 551–561. http://doi.org/10.1016/j.euroneuro.2009.02.003

Davis, J. A., James, J. R., Siegel, S. J., & Gould, T. J. (2005). Withdrawal from Chronic Nicotine Administration Impairs Contextual Fear Conditioning in C57BL/6 Mice. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *25*(38), 8708–8713. http://doi.org/10.1523/JNEUROSCI.2853-05.2005

Davis, J. A., Kenney, J. W., & Gould, T. J. (2007). Hippocampal α4β2 Nicotinic Acetylcholine Receptor Involvement in the Enhancing Effect of Acute Nicotine on Contextual Fear Conditioning. *The Journal of Neuroscience*, *27*(40), 10870–10877. http://doi.org/10.1523/JNEUROSCI.3242-07.2007

Durazzo, T. C., Meyerhoff, D. J., & Nixon, S. J. (2010). Chronic Cigarette Smoking: Implications for Neurocognition and Brain Neurobiology. *International Journal of Environmental Research and Public Health*, *7*(10), 3760–3791. http://doi.org/10.3390/ijerph7103760

Elias, G. A., Gulick, D., Wilkinson, D. S., & Gould, T. J. (2010). Nicotine and extinction of fear conditioning. *Neuroscience*, *165*(4), 1063–1073. http://doi.org/10.1016/j.neuroscience.2009.11.022

Elzinga, B. M., & Bremner, J. D. (2002). Are the neural substrates of memory the final common pathway in posttraumatic stress disorder (PTSD)? *Journal of Affective Disorders*, *70*(1), 1–17. http://doi.org/10.1016/S0165-0327(01)00351-2

Eppolito, A. K., Bachus, S. E., McDonald, C. G., Meador-Woodruff, J. H., & Smith, R. F. (2010). Late emerging effects of prenatal and early postnatal nicotine exposure on the cholinergic system and anxiety-like behavior. *Neurotoxicology and Teratology*, *32*(3), 336–345. http://doi.org/10.1016/j.ntt.2009.12.009

Everitt, B. J., & Robbins, T. W. (1997). Central Cholinergic Systems and Cognition. *Annual Review of Psychology*, *48*(1), 649–684. http://doi.org/10.1146/annurev.psych.48.1.649

Fanselow, M. S. (2010). From contextual fear to a dynamic view of memory systems. *Trends in Cognitive Sciences*, *14*(1), 7–15. http://doi.org/10.1016/j.tics.2009.10.008

Fanselow, M. S., & Dong, H.-W. (2010). Are the dorsal and ventral hippocampus functionally distinct structures? *Neuron*, *65*(1), 7–19. http://doi.org/10.1016/j.neuron.2009.11.031

Feldner, M. T., Babson, K. A., & Zvolensky, M. J. (2007). Smoking, traumatic event exposure, and post-traumatic stress: A critical review of the empirical literature. *Clinical Psychology Review*, *27*(1), 14–45. http://doi.org/10.1016/j.cpr.2006.08.004

Fendt, M., Fanselow, M. S., & Koch, M. (2005). Lesions of the Dorsal Hippocampus Block Trace Fear Conditioned Potentiation of Startle. *Behavioral Neuroscience*, *119*(3), 834–838. http://doi.org/10.1037/0735-7044.119.3.834

Fenster, C. P., Rains, M. F., Noerager, B., Quick, M. W., & Lester, R. A. J. (1997). Influence of Subunit Composition on Desensitization of Neuronal Acetylcholine Receptors at Low Concentrations of Nicotine. *The Journal of Neuroscience*, *17*(15), 5747–5759.

Franklin, J. L., Fickbohm, D. J., & Willard, A. L. (1992). Long-term regulation of neuronal calcium currents by prolonged changes of membrane potential. *The Journal of Neuroscience*, *12*(5), 1726–1735.

Fujii, S., Jia, Y., Yang, A., & Sumikawa, K. (2000). Nicotine reverses GABAergic inhibition of long-term potentiation induction in the hippocampal CA1 region. *Brain Research*, *863*(1–2), 259–265. http://doi.org/10.1016/S0006-8993(00)02119-3

Gamwell, K., Nylocks, M., Cross, D., Bradley, B., Norrholm, S. D., & Jovanovic, T. (2015). Fear conditioned responses and PTSD symptoms in children: Sex differences in fear-related symptoms. *Developmental Psychobiology*, *57*(7), 799–808. http://doi.org/10.1002/dev.21313

Gerber, B., Yarali, A., Diegelmann, S., Wotjak, C. T., Pauli, P., & Fendt, M. (2014). Pain-relief learning in flies, rats, and man: basic research and applied perspectives. *Learning & Memory*, *21*(4), 232–252. http://doi.org/10.1101/lm.032995.113

Ge, S., & Dani, J. A. (2005). Nicotinic Acetylcholine Receptors at Glutamate Synapses Facilitate Long-Term Depression or Potentiation. *The Journal of Neuroscience*, *25*(26), 6084–6091. http://doi.org/10.1523/JNEUROSCI.0542-05.2005

Gewirtz, J. C., Falls, W. A., & Davis, M. (1997). Normal conditioning inhibition and extinction of freezing and fear-potentiated startle following electrolytic lesions of medial prefrontal cortex in rats. *Behavioral Neuroscience*, *111*(4), 712–726. http://doi.org/10.1037/0735-7044.111.4.712

Gilmartin, M. R., Kwapis, J. L., & Helmstetter, F. J. (2012). Trace and contextual fear conditioning are impaired following unilateral microinjection of muscimol in the ventral hippocampus or amygdala, but not the medial prefrontal cortex. *Neurobiology of Learning and Memory*, *97*(4), 452–464. http://doi.org/10.1016/j.nlm.2012.03.009

Gilmartin, M. R., Kwapis, J. L., & Helmstetter, F. J. (2013). NR2A- and NR2B-containing NMDA receptors in the prelimbic medial prefrontal cortex differentially mediate trace, delay, and contextual fear conditioning. *Learning & Memory*, *20*(6), 290–294. http://doi.org/10.1101/lm.030510.113

Gilmartin, M. R., & McEchron, M. D. (2005). Single neurons in the medial prefrontal cortex of the rat exhibit tonic and phasic coding during trace fear conditioning. *Behavioral Neuroscience*, *119*(6), 1496–1510. http://doi.org/10.1037/0735-7044.119.6.1496

Gilmartin, M. R., Miyawaki, H., Helmstetter, F. J., & Diba, K. (2013). Prefrontal Activity Links Nonoverlapping Events in Memory. *The Journal of Neuroscience*, *33*(26), 10910–10914. http://doi.org/10.1523/JNEUROSCI.0144-13.2013

Gould, T. J. (2006). Nicotine and Hippocampus-Dependent Learning. *Molecular Neurobiology*, *34*(2), 93–107. http://doi.org/10.1385/MN:34:2:93

Gould, T. J., Feiro, O., & Moore, D. (2004). Nicotine enhances trace cued fear conditioning but not delay cued fear conditioning in C57BL/6 mice. *Behavioural Brain Research*, *155*(1), 167–173. http://doi.org/10.1016/j.bbr.2004.04.009

Gould, T. J., & Higgins, J. S. (2003). Nicotine enhances contextual fear conditioning in C57BL/6J mice at 1 and 7 days post-training. *Neurobiology of Learning and Memory*, *80*(2), 147–157. http://doi.org/10.1016/S1074-7427(03)00057-1

Gould, T. J., McCarthy, M. M., & Keith, R. A. (2002). MK-801 disrupts acquisition of contextual fear conditioning but enhances memory consolidation of cued fear conditioning. *Behavioural Pharmacology*, *13*(4), 287–294.

Gould, T. J., Portugal, G. S., André, J. M., Tadman, M. P., Marks, M. J., Kenney, J. W., … Adoff, M. (2012a). The duration of nicotine withdrawal-associated deficits in contextual fear conditioning parallels changes in hippocampal high affinity nicotinic acetylcholine receptor upregulation. *Neuropharmacology*, *62*(5–6), 2118–2125. http://doi.org/10.1016/j.neuropharm.2012.01.003

Gould, T. J., Portugal, G. S., André, J. M., Tadman, M. P., Marks, M. J., Kenney, J. W., … Adoff, M. (2012b). The duration of nicotine withdrawal-associated deficits in contextual fear conditioning parallels changes in hippocampal high affinity nicotinic acetylcholine receptor upregulation. *Neuropharmacology*, *62*(5–6), 2118–2125. http://doi.org/10.1016/j.neuropharm.2012.01.003

Gould, T. J., Wilkinson, D. S., Yildirim, E., Blendy, J. A., & Adoff, M. D. (2014). Dissociation of tolerance and nicotine withdrawal-associated deficits in contextual fear. *Brain Research*, *1559*, 1–10. http://doi.org/10.1016/j.brainres.2014.02.038

Gould, T. J., Wilkinson, D. S., Yildirim, E., Poole, R. L. F., Leach, P. T., & Simmons, S. J. (2014). Nicotine shifts the temporal activation of hippocampal protein kinase A and extracellular signal-regulated kinase 1/2 to enhance long-term, but not short-term, hippocampus-dependent memory. *Neurobiology of Learning and Memory*, *109*, 151–159. http://doi.org/10.1016/j.nlm.2014.01.009

Granon, S., Poucet, B., Thinus-Blanc, C., Changeux, J.-P., & Vidal, C. (1995). Nicotinic and muscarinic receptors in the rat prefrontal cortex: Differential roles in working memory, response selection and effortful processing. *Psychopharmacology*, *119*(2), 139–144. http://doi.org/10.1007/BF02246154

Guan, X., Nakauchi, S., & Sumikawa, K. (2006). Nicotine reverses consolidated long-term potentiation in the hippocampal CA1 region. *Brain Research*, *1078*(1), 80–91. http://doi.org/10.1016/j.brainres.2006.02.034

Guillem, K., Bloem, B., Poorthuis, R. B., Loos, M., Smit, A. B., Maskos, U., … Mansvelder, H. D. (2011). Nicotinic Acetylcholine Receptor β2 Subunits in the Medial Prefrontal Cortex Control Attention. *Science*, *333*(6044), 888–891. http://doi.org/10.1126/science.1207079

Gu, Z., Lamb, P. W., & Yakel, J. L. (2012). Cholinergic Coordination of Presynaptic and Postsynaptic Activity Induces Timing-Dependent Hippocampal Synaptic Plasticity. *The Journal of Neuroscience*, *32*(36), 12337–12348. http://doi.org/10.1523/JNEUROSCI.2129-12.2012

Hasselmo, M. E. (2006). The Role of Acetylcholine in Learning and Memory. *Current Opinion in Neurobiology*, *16*(6), 710–715. http://doi.org/10.1016/j.conb.2006.09.002

Heldt, S. A., Coover, G. D., & Falls, W. A. (2002). Posttraining but not pretraining lesions of the hippocampus interfere with feature-negative discrimination of fear-potentiated startle. *Hippocampus*, *12*(6), 774–786. http://doi.org/10.1002/hipo.10033

Hendin, H., & Haas, A. P. (1991). Suicide and guilt as manifestations of PTSD in Vietnam combat veterans. *The American Journal of Psychiatry*, *148*(5), 586–591.

Honey, R. C., Watt, A., & Good, M. (1998). Hippocampal Lesions Disrupt an Associative Mismatch Process. *The Journal of Neuroscience*, *18*(6), 2226–2230.

Hoover, W. B., & Vertes, R. P. (2007). Anatomical analysis of afferent projections to the medial prefrontal cortex in the rat. *Brain Structure and Function*, *212*(2), 149–179. http://doi.org/10.1007/s00429-007-0150-4

Horsley, R. R., & Cassaday, H. J. (2007). Methylphenidate can reduce selectivity in associative learning in an aversive trace conditioning task. *Journal of Psychopharmacology*, *21*(5), 492–500. http://doi.org/10.1177/0269881106067381

Inslicht, S. S., Metzler, T. J., Garcia, N. M., Pineles, S. L., Milad, M. R., Orr, S. P., … Neylan, T. C. (2013). Sex differences in fear conditioning in posttraumatic stress disorder. *Journal of Psychiatric Research*, *47*(1), 64–71. http://doi.org/10.1016/j.jpsychires.2012.08.027

Ji, D., & Dani, J. A. (2000). Inhibition and Disinhibition of Pyramidal Neurons by Activation of Nicotinic Receptors on Hippocampal Interneurons. *Journal of Neurophysiology*, *83*(5), 2682–2690.

Ji, D., Lape, R., & Dani, J. A. (2001). Timing and Location of Nicotinic Activity Enhances or Depresses Hippocampal Synaptic Plasticity. *Neuron*, *31*(1), 131–141. http://doi.org/10.1016/S0896-6273(01)00332-4

Jovanovic, T., Kazama, A., Bachevalier, J., & Davis, M. (2012). Impaired safety signal learning may be a biomarker of PTSD. *Neuropharmacology*, *62*(2), 695–704. http://doi.org/10.1016/j.neuropharm.2011.02.023

Jovanovic, T., Norrholm, S. D., Blanding, N. Q., Davis, M., Duncan, E., Bradley, B., & Ressler, K. J. (2010). Impaired fear inhibition is a biomarker of PTSD but not depression. *Depression and Anxiety*, *27*(3), 244–251. http://doi.org/10.1002/da.20663

Jovanovic, T., Norrholm, S. D., Blanding, N. Q., Phifer, J. E., Weiss, T., Davis, M., … Ressler, K. (2010). Fear potentiation is associated with hypothalamic–pituitary–adrenal axis function in PTSD. *Psychoneuroendocrinology*, *35*(6), 846–857. http://doi.org/10.1016/j.psyneuen.2009.11.009

Jovanovic, T., Norrholm, S. D., Fennell, J. E., Keyes, M., Fiallos, A. M., Myers, K. M., … Duncan, E. J. (2009). Posttraumatic stress disorder may be associated with impaired fear inhibition: Relation to symptom severity. *Psychiatry Research*, *167*(1–2), 151–160. http://doi.org/10.1016/j.psychres.2007.12.014

Jovanovic, T., Nylocks, K. M., Gamwell, K. L., Smith, A., Davis, T. A., Norrholm, S. D., & Bradley, B. (2014). Development of fear acquisition and extinction in children: Effects of age and anxiety. *Neurobiology of Learning and Memory*, *113*, 135–142. http://doi.org/10.1016/j.nlm.2013.10.016

Katz, B., & Thesleff, S. (1957). A study of the `desensitization' produced by acetylcholine at the motor end-plate. *The Journal of Physiology*, *138*(1), 63–80.

Kazama, A. M., Heuer, E., Davis, M., & Bachevalier, J. (2012). Effects of neonatal amygdala lesions on fear learning, conditioned inhibition, and extinction in adult macaques. *Behavioral Neuroscience*, *126*(3), 392–403. http://doi.org/10.1037/a0028241

Kenney, J. W., Adoff, M. D., Wilkinson, D. S., & Gould, T. J. (2011). The effects of acute, chronic, and withdrawal from chronic nicotine on novel and spatial object recognition in male C57BL/6J mice. *Psychopharmacology*, *217*(3), 353–365. http://doi.org/10.1007/s00213-011-2283-7

Kenney, J. W., Poole, R. L., Adoff, M. D., Logue, S. F., & Gould, T. J. (2012). Learning and Nicotine Interact to Increase CREB Phosphorylation at the jnk1 Promoter in the Hippocampus. *PLoS ONE*, *7*(6), e39939. http://doi.org/10.1371/journal.pone.0039939

Kenney, J. W., Raybuck, J. D., & Gould, T. J. (2012a). Nicotinic receptors in the dorsal and ventral hippocampus differentially modulate contextual fear conditioning. *Hippocampus*, *22*(8), 1681–1690. http://doi.org/10.1002/hipo.22003

Kenney, J. W., Raybuck, J. D., & Gould, T. J. (2012b). Nicotinic receptors in the dorsal and ventral hippocampus differentially modulate contextual fear conditioning. *Hippocampus*, *22*(8), 1681–1690. http://doi.org/10.1002/hipo.22003

Kessler, R. C., Borges, G., & Walters, E. E. (1999). Prevalence of and risk factors for lifetime suicide attempts in the National Comorbidity Survey. *Archives of General Psychiatry*, *56*(7), 617–626.

Kim, J. J., Rison, R. A., & Fanselow, M. S. (1993). Effects of amygdala, hippocampus, and periaqueductal gray lesions on short- and long-term contextual fear. *Behavioral Neuroscience*, *107*(6), 1093–1098. http://doi.org/10.1037/0735-7044.107.6.1093

Kjelstrup, K. G., Tuvnes, F. A., Steffenach, H.-A., Murison, R., Moser, E. I., & Moser, M.-B. (2002). Reduced fear expression after lesions of the ventral hippocampus. *Proceedings of the National Academy of Sciences*, *99*(16), 10825–10830. http://doi.org/10.1073/pnas.152112399

Klopf, A. H. (1988). A neuronal model of classical conditioning. *Psychobiology*, *16*(2), 85–125. http://doi.org/10.3758/BF03333113

Kong, E., Monje, F. J., Hirsch, J., & Pollak, D. D. (2014). Learning not to Fear: Neural Correlates of Learned Safety. *Neuropsychopharmacology*, *39*(3), 515–527. http://doi.org/10.1038/npp.2013.191

Kutlu, M. G., & Gould, T. J. (2014). Acute nicotine delays extinction of contextual fear in mice. *Behavioural Brain Research*, *263*, 133–137. http://doi.org/10.1016/j.bbr.2014.01.031

Kutlu, M. G., Oliver, C., & Gould, T. J. (2014). The effects of acute nicotine on contextual safety discrimination. *Journal of Psychopharmacology*, *28*(11), 1064–1070. http://doi.org/10.1177/0269881114552743

Lapierre, C. B. (2008). Deployment with combat exposure increases the risk of new-onset PTSD. *Evidence Based Mental Health*, *11*(4), 126–126. http://doi.org/10.1136/ebmh.11.4.126

Leach, P. T., Kenney, J. W., Connor, D. A., & Gould, T. J. (2015). Thyroid receptor β involvement in the effects of acute nicotine on hippocampus-dependent memory. *Neuropharmacology*, *93*, 155–163. http://doi.org/10.1016/j.neuropharm.2015.01.026

Levin, E. D., Lee, C., Rose, J. E., Reyes, A., Ellison, G., Jarvik, M., & Gritz, E. (1990). Chronic nicotine and withdrawal effects on radial-arm maze performance in rats. *Behavioral and Neural Biology*, *53*(2), 269–276. http://doi.org/10.1016/0163-1047(90)90509-5

Levin, E. D., & Rose, J. E. (1991). Nicotinic and muscarinic interactions and choice accuracy in the radial-arm maze. *Brain Research Bulletin*, *27*(1), 125–128. http://doi.org/10.1016/0361-9230(91)90293-S

Levin, E. D., & Torry, D. (1996). Acute and chronic nicotine effects on working memory in aged rats. *Psychopharmacology*, *123*(1), 88–97. http://doi.org/10.1007/BF02246285

Liao, B., & Craske, M. G. (2013). The Impact of State Anxiety on Fear Inhibition. *Journal of Experimental Psychopathology*. http://doi.org/10.5127/jep.026612

Liberzon, I., & Sripada, C. S. (2007). The functional neuroanatomy of PTSD: a critical review. In M. S. O. and E. V. E. Ronald De Kloet (Ed.), *Progress in Brain Research* (Vol. 167, pp. 151–169). Elsevier. Retrieved from http://www.sciencedirect.com/science/article/pii/S0079612307670113

Liu, I. Y. C., Lyons, W. E., Mamounas, L. A., & Thompson, R. F. (2004). Brain-Derived Neurotrophic Factor Plays a Critical Role in Contextual Fear Conditioning. *The Journal of Neuroscience*, *24*(36), 7958–7963. http://doi.org/10.1523/JNEUROSCI.1948-04.2004

Logue, S. F., Paylor, R., & Wehner, J. M. (1997). Hippocampal lesions cause learning deficits in inbred mice in the Morris water maze and conditioned-fear task. *Behavioral Neuroscience*, *111*(1), 104–113. http://doi.org/10.1037/0735-7044.111.1.104

Lu, C., Li, C., Li, D., & Henderson, Z. (2013). Nicotine induction of theta frequency oscillations in rodent medial septal diagonal band in vitro. *Acta Pharmacologica Sinica*, *34*(6), 819–829. http://doi.org/10.1038/aps.2012.198

Maggio, N., & Segal, M. (2009). Differential Modulation of Long-Term Depression by Acute Stress in the Rat Dorsal and Ventral Hippocampus. *The Journal of Neuroscience*, *29*(27), 8633–8638. http://doi.org/10.1523/JNEUROSCI.1901-09.2009

Maren, S. (2008). Pavlovian fear conditioning as a behavioral assay for hippocampus and amygdala function: cautions and caveats. *European Journal of Neuroscience*, *28*(8), 1661–1666. http://doi.org/10.1111/j.1460-9568.2008.06485.x

Maren, S., & Fanselow, M. S. (1995). Synaptic plasticity in the basolateral amygdala induced by hippocampal formation stimulation in vivo. *The Journal of Neuroscience*, *15*(11), 7548–7564.

Markou, A. (2008). Neurobiology of nicotine dependence. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *363*(1507), 3159–3168.

Marks, M. J., Grady, S. R., & Collins, A. C. (1993). Downregulation of nicotinic receptor function after chronic nicotine infusion. *The Journal of Pharmacology and Experimental Therapeutics*, *266*(3), 1268–1276.

Marks, M. J., Pauly, J. R., Gross, S. D., Deneris, E. S., Hermans-Borgmeyer, I., Heinemann, S. F., & Collins, A. C. (1992). Nicotine binding and nicotinic receptor subunit RNA after chronic nicotine treatment. *The Journal of Neuroscience*, *12*(7), 2765–2784.

Marks, M. J., Romm, E., Campbell, S. M., & Collins, A. C. (1989). Variation of nicotinic binding sites among inbred strains. *Pharmacology Biochemistry and Behavior*, *33*(3), 679–689. http://doi.org/10.1016/0091-3057(89)90407-3

Marlin, N. A. (1982). Within-compound associations between the context and the conditioned stimulus. *Learning and Motivation*, *13*(4), 526–541. http://doi.org/10.1016/0023-9690(82)90008-X

Matsuyama, S., Matsumoto, A., Enomoto, T., & Nishizaki, T. (2000). Activation of nicotinic acetylcholine receptors induces long-term potentiation in vivo in the intact mouse dentate gyrus. *European Journal of Neuroscience*, *12*(10), 3741–3747. http://doi.org/10.1046/j.1460-9568.2000.00259.x

McNally, G. P., & Westbrook, R. F. (2006). Predicting danger: The nature, consequences, and neural mechanisms of predictive fear learning. *Learning & Memory*, *13*(3), 245–253. http://doi.org/10.1101/lm.196606

Mohammadi, M., Bergado-Acosta, J. R., & Fendt, M. (2014). Relief learning is distinguished from safety learning by the requirement of the nucleus accumbens. *Behavioural Brain Research*, *272*, 40–45. http://doi.org/10.1016/j.bbr.2014.06.053

Moyer, J. R., Deyo, R. A., & Disterhoft, J. F. (1990). Hippocampectomy disrupts trace eye-blink conditioning in rabbits. *Behavioral Neuroscience*, *104*(2), 243–252. http://doi.org/10.1037/0735-7044.104.2.243

Myers, C. E., & Scharfman, H. E. (2011). Pattern Separation in the Dentate Gyrus: A Role for the CA3 Backprojection. *Hippocampus*, *21*(11), 1190–1215. http://doi.org/10.1002/hipo.20828

Nakayama, H., Numakawa, T., Ikeuchi, T., & Hatanaka, H. (2001). Nicotine-induced phosphorylation of extracellular signal-regulated protein kinase and CREB in PC12h cells. *Journal of Neurochemistry*, *79*(3), 489–498. http://doi.org/10.1046/j.1471-4159.2001.00602.x

Norman, C., & Cassaday, H. J. (2003). Amphetamine increases aversive conditioning to diffuse contextual stimuli and to a discrete trace stimulus when conditioned at higher footshock intensity. *Journal of Psychopharmacology*, *17*(1), 67–76. http://doi.org/10.1177/0269881103017001701

Parikh, V., Man, K., Decker, M. W., & Sarter, M. (2008). Glutamatergic Contributions to Nicotinic Acetylcholine Receptor Agonist-Evoked Cholinergic Transients in the Prefrontal Cortex. *The Journal of Neuroscience*, *28*(14), 3769–3780. http://doi.org/10.1523/JNEUROSCI.5251-07.2008

Phillips, R. G., & LeDoux, J. E. (1992). Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. *Behavioral Neuroscience*, *106*(2), 274–285.

Phillips, R. G., & LeDoux, J. E. (1994). Lesions of the dorsal hippocampal formation interfere with background but not foreground contextual fear conditioning. *Learning & Memory*, *1*(1), 34–44. http://doi.org/10.1101/lm.1.1.34

Picciotto, M. R. (2003). Nicotine as a modulator of behavior: beyond the inverted U. *Trends in Pharmacological Sciences*, *24*(9), 493–499. http://doi.org/10.1016/S0165-6147(03)00230-X

Pitkänen, A., Pikkarainen, M., Nurminen, N., & Ylinen, A. (2000). Reciprocal Connections between the Amygdala and the Hippocampal Formation, Perirhinal Cortex, and Postrhinal Cortex in Rat: A Review. *Annals of the New York Academy of Sciences*, *911*(1), 369–391. http://doi.org/10.1111/j.1749-6632.2000.tb06738.x

Placzek, A. N., Zhang, T. A., & Dani, J. A. (2009). Nicotinic mechanisms influencing synaptic plasticity in the hippocampus. *Acta Pharmacologica Sinica*, *30*(6), 752–760. http://doi.org/10.1038/aps.2009.39

Pole, N., Neylan, T. C., Otte, C., Henn-Hasse, C., Metzler, T. J., & Marmar, C. R. (2009). Prospective Prediction of PTSD Symptoms Using Fear Potentiated Auditory Startle Responses. *Biological Psychiatry*, *65*(3), 235–240. http://doi.org/10.1016/j.biopsych.2008.07.015

Pollak, D. D., Monje, F. J., & Lubec, G. (2010). The learned safety paradigm as a mouse model for neuropsychiatric research. *Nature Protocols*, *5*(5), 954–962. http://doi.org/10.1038/nprot.2010.64

Pollak, D. D., Monje, F. J., Zuckerman, L., Denny, C. A., Drew, M. R., & Kandel, E. R. (2008). An Animal Model of a Behavioral Intervention for Depression. *Neuron*, *60*(1), 149–161. http://doi.org/10.1016/j.neuron.2008.07.041

Poole, R. L., Connor, D. A., & Gould, T. J. (2014). Donepezil Reverses Nicotine Withdrawal-Induced Deficits in Contextual Fear Conditioning in C57BL/6J Mice. *Behavioral Neuroscience*. http://doi.org/10.1037/bne0000003

Portugal, G. S., Wilkinson, D. S., Kenney, J. W., Sullivan, C., & Gould, T. J. (2012). Strain-dependent Effects of Acute, Chronic, and Withdrawal from Chronic Nicotine on Fear Conditioning. *Behavior Genetics*, *42*(1), 133–150. http://doi.org/10.1007/s10519-011-9489-7

Portugal, G. S., Wilkinson, D. S., Turner, J. R., Blendy, J. A., & Gould, T. J. (2012). Developmental effects of acute, chronic, and withdrawal from chronic nicotine on fear conditioning. *Neurobiology of Learning and Memory*, *97*(4), 482–494. http://doi.org/10.1016/j.nlm.2012.04.003

Provost, S. C., & Woodward, R. (1991). Effects of nicotine gum on repeated administration of the stroop test. *Psychopharmacology*, *104*(4), 536–540. http://doi.org/10.1007/BF02245662

Quick, M. W., & Lester, R. A. J. (2002). Desensitization of neuronal nicotinic receptors. *Journal of Neurobiology*, *53*(4), 457–478. http://doi.org/10.1002/neu.10109

Quinn, J. J., Loya, F., Ma, Q. D., & Fanselow, M. S. (2005). Dorsal hippocampus NMDA receptors differentially mediate trace and contextual fear conditioning. *Hippocampus*, *15*(5), 665–674. http://doi.org/10.1002/hipo.20088

Quinn, J. J., Oommen, S. S., Morrison, G. E., & Fanselow, M. S. (2002). Post-training excitotoxic lesions of the dorsal hippocampus attenuate forward trace, backward trace, and delay fear conditioning in a temporally specific manner. *Hippocampus*, *12*(4), 495–504. http://doi.org/10.1002/hipo.10029

Radcliffe, K. A., Fisher, J. L., Gray, R., & Dani, J. A. (1999). Nicotinic Modulation of Glutamate and GABA Synaptic Transmission in Hippocampal Neurons. *Annals of the New York Academy of Sciences*, *868*(1), 591–610. http://doi.org/10.1111/j.1749-6632.1999.tb11332.x

Ragozzino, M. E., Detrick, S., & Kesner, R. P. (2002). The Effects of Prelimbic and Infralimbic Lesions on Working Memory for Visual Objects in Rats. *Neurobiology of Learning and Memory*, *77*(1), 29–43. http://doi.org/10.1006/nlme.2001.4003

Raybuck, J. D., & Gould, T. J. (2009). Nicotine Withdrawal-Induced Deficits in Trace Fear Conditioning in C57BL/6 Mice: A Role for High-Affinity ?2 Subunit-Containing Nicotinic Acetylcholine Receptors. *The European Journal of Neuroscience*, *29*(2), 377–387. http://doi.org/10.1111/j.1460-9568.2008.06580.x

Raybuck, J. D., & Gould, T. J. (2010). The role of nicotinic acetylcholine receptors in the medial prefrontal cortex and hippocampus in trace fear conditioning. *Neurobiology of Learning and Memory*, *94*(3), 353–363. http://doi.org/10.1016/j.nlm.2010.08.001

Raybuck, J. D., & Lattal, K. M. (2014). Bridging the interval: Theory and neurobiology of trace conditioning. *Behavioural Processes*, *101*, 103–111. http://doi.org/10.1016/j.beproc.2013.08.016

Raybuck, J. D., Portugal, G. S., Lerman, C., & Gould, T. J. (2008). Varenicline Ameliorates Nicotine Withdrawal-Induced Learning Deficits in C57BL/6 Mice. *Behavioral Neuroscience*, *122*(5), 1166–1171. http://doi.org/10.1037/a0012601

Rescorla, R. A. (1969). Pavlovian conditioned inhibition. *Psychological Bulletin*, *72*(2), 77–94. http://doi.org/10.1037/h0027760

Ressler, K. J., Mercer, K. B., Bradley, B., Jovanovic, T., Mahan, A., Kerley, K., … May, V. (2011). Post-traumatic stress disorder is associated with PACAP and the PAC1 receptor. *Nature*, *470*(7335), 492–497. http://doi.org/10.1038/nature09856

Rezvani, A. H., & Levin, E. D. (2001). Cognitive effects of nicotine. *Biological Psychiatry*, *49*(3), 258–267. http://doi.org/10.1016/S0006-3223(00)01094-5

Rothbaum, B. O., & Davis, M. (2003). Applying Learning Principles to the Treatment of Post-Trauma Reactions. *Annals of the New York Academy of Sciences*, *1008*(1), 112–121. http://doi.org/10.1196/annals.1301.012

Rougemont-Bücking, A., Linnman, C., Zeffiro, T. A., Zeidan, M. A., Lebron-Milad, K., Rodriguez-Romaguera, J., … Milad, M. R. (2011). Altered Processing of Contextual Information during Fear Extinction in PTSD: An fMRI Study. *CNS Neuroscience & Therapeutics*, *17*(4), 227–236. http://doi.org/10.1111/j.1755-5949.2010.00152.x

Sarter, M., Parikh, V., & Howe, W. M. (2009). nAChR agonist-induced cognition enhancement: Integration of cognitive and neuronal mechanisms. *Biochemical Pharmacology*, *78*(7), 658–667. http://doi.org/10.1016/j.bcp.2009.04.019

Sawada, S., Yamamoto, C., & Ohno-Shosaku, T. (1994). Long-term potentiation and depression in the dentate gyrus, and effects of nicotine. *Neuroscience Research*, *20*(4), 323–329. http://doi.org/10.1016/0168-0102(94)90054-X

Schiller, D., Levy, I., Niv, Y., LeDoux, J. E., & Phelps, E. A. (2008). From Fear to Safety and Back: Reversal of Fear in the Human Brain. *The Journal of Neuroscience*, *28*(45), 11517–11525. http://doi.org/10.1523/JNEUROSCI.2265-08.2008

Shearman, E., Rossi, S., Sershen, H., Hashim, A., & Lajtha, A. (2005). Locally Administered Low Nicotine-Induced Neurotransmitter Changes in Areas of Cognitive Function. *Neurochemical Research*, *30*(8), 1055–1066. http://doi.org/10.1007/s11064-005-7132-9

Shors, T. J. (2004). Memory traces of trace memories: neurogenesis, synaptogenesis and awareness. *Trends in Neurosciences*, *27*(5), 250–256. http://doi.org/10.1016/j.tins.2004.03.007

Shors, T. J., Miesegaes, G., Beylin, A., Zhao, M., Rydel, T., & Gould, E. (2001). Neurogenesis in the adult is involved in the formation of trace memories. *Nature*, *410*(6826), 372–376. http://doi.org/10.1038/35066584

Sierra-Mercado, D., Padilla-Coreano, N., & Quirk, G. J. (2011). Dissociable Roles of Prelimbic and Infralimbic Cortices, Ventral Hippocampus, and Basolateral Amygdala in the Expression and Extinction of Conditioned Fear. *Neuropsychopharmacology*, *36*(2), 529–538. http://doi.org/10.1038/npp.2010.184

Smith, B., Ryan, M. A. K., Wingard, D. L., Patterson, T. L., Slymen, D. J., & Macera, C. A. (2008). Cigarette Smoking and Military Deployment: A Prospective Evaluation. *American Journal of Preventive Medicine*, *35*(6), 539–546. http://doi.org/10.1016/j.amepre.2008.07.009

Squire, L. R., & Zola, S. M. (1996). Structure and function of declarative and nondeclarative memory systems. *Proceedings of the National Academy of Sciences*, *93*(24), 13515–13522.

Talhout, R., Schulz, T., Florek, E., van Benthem, J., Wester, P., & Opperhuizen, A. (2011). Hazardous Compounds in Tobacco Smoke. *International Journal of Environmental Research and Public Health*, *8*(2), 613–628. http://doi.org/10.3390/ijerph8020613

Tang, J., & Dani, J. A. (2009). Dopamine Enables In Vivo Synaptic Plasticity Associated with the Addictive Drug Nicotine. *Neuron*, *63*(5), 673–682. http://doi.org/10.1016/j.neuron.2009.07.025

Tarrier, P. N., & Gregg, L. (2004). Suicide risk in civilian PTSD patients. *Social Psychiatry and Psychiatric Epidemiology*, *39*(8), 655–661. http://doi.org/10.1007/s00127-004-0799-4

Valentino, R. J., Bangasser, D., & Van Bockstaele, E. J. (2013). Sex-biased stress signaling: the corticotropin-releasing factor receptor as a model. *Molecular Pharmacology*, *83*(4), 737–745. http://doi.org/10.1124/mol.112.083550

VanElzakker, M. B., Kathryn Dahlgren, M., Caroline Davis, F., Dubois, S., & Shin, L. M. (2014). From Pavlov to PTSD: The extinction of conditioned fear in rodents, humans, and anxiety disorders. *Neurobiology of Learning and Memory*, *113*, 3–18. http://doi.org/10.1016/j.nlm.2013.11.014

Velden, P. G. V. der, Grievink, L., Olff, M., Gersons, B. P. R., & Kleber, R. J. (2007). Smoking as a Risk Factor for Mental Health Disturbances After a Disaster: A Prospective Comparative Study. *The Journal of Clinical Psychiatry*, *68*(1), 87–92.

Vertes, R. P. (2004). Differential projections of the infralimbic and prelimbic cortex in the rat. *Synapse*, *51*(1), 32–58. http://doi.org/10.1002/syn.10279

Welsby, P. J. R. Michael J.Anwyl, Roger. (2009). Intracellular mechanisms underlying the nicotinic enhancement of LTP in the rat dentate gyrus. *European Journal of Neuroscience*, *29*(1), 65–75. http://doi.org/10.1111/j.1460-9568.2008.06562.x

Williams, D. A., Overmier, J. B., & LoLordo, V. M. (1992). A reevaluation of Rescorla's early dictums about Pavlovian conditioned inhibition. *Psychological Bulletin*, *111*(2), 275–290. http://doi.org/10.1037/0033-2909.111.2.275

Woodruff-Pak, D. S. (2003). Mecamylamine reversal by nicotine and by a partial α7 nicotinic acetylcholine receptor agonist (GTS-21) in rabbits tested with delay eyeblink classical conditioning. *Behavioural Brain Research*, *143*(2), 159–167. http://doi.org/10.1016/S0166-4328(03)00039-1

Woodruff-Pak, D. S., & Disterhoft, J. F. (2008). Where is the trace in trace conditioning? *Trends in Neurosciences*, *31*(2), 105–112. http://doi.org/10.1016/j.tins.2007.11.006

Yang, K., & Dani, J. A. (2014). Dopamine D1 and D5 Receptors Modulate Spike Timing-Dependent Plasticity at Medial Perforant Path to Dentate Granule Cell Synapses. *The Journal of Neuroscience*, *34*(48), 15888–15897. http://doi.org/10.1523/JNEUROSCI.2400-14.2014

Yarali, A., Nehrkorn, J., Tanimoto, H., & Herz, A. V. M. (2012). Event Timing in Associative Learning: From Biochemical Reaction Dynamics to Behavioural Observations. *PLoS ONE*, *7*(3), e32885. http://doi.org/10.1371/journal.pone.0032885