SPECIFIC EFFECTS OF NICOTINE AND NICOTINIC ANTAGONISTS ON TRACE AND CONTEXUTAL FEAR CONDITIONING IN C57BL/6 MICE: A ROLE FOR NICOTINIC ACETYLCHOLINERGIC SIGNALING IN THE DORSAL HIPPOCAMPUS, VENTRAL HIPPOCAMPUS, AND MEDIAL PREFRONTAL CORTEX IN TRACE FEAR CONDITIONING

A Dissertation
Submitted to the
Temple University Graduate Board

In Partial Fulfillment of
The Requirements for the Degree
DOCTOR OF PHILOSOPHY

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January 2010

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ABSTRACT

Nicotine has been shown to enhance multiple forms of learning and memory. However the substrates through which these effects occur are not well understood. To examine the specific substrates of nicotine's acute effects on trace fear conditioning, I infused nicotine into areas thought to support trace fear conditioning, the dorsal hippocampus, ventral hippocampus and medial prefrontal cortex. Additionally, we investigated the contributions of nicotinic acetylcholinergic signaling to trace fear conditioning by infusing the nicotinic antagonists dihydro-beta-erythroidine (DHβE) and methyllycaconitine (MLA) into these areas. Nicotine had different effects on both trace and contextual fear conditioning depending on dose and brain region, as did the nicotinic antagonists. In the dorsal hippocampus nicotine infusion enhanced both trace and contextual conditioning, although these effects were dissociable by dose and training protocol. Additionally, the high-affinity nicotinic antagonist DHβE produced selective deficits in trace conditioning, suggesting that while enhancement of nicotinic signaling can affect both contextual and trace learning, nicotinic activity in the dorsal hippocampus is critically involved in trace but not contextual conditioning. In the ventral hippocampus nicotine infusion produced deficits in both trace and contextual fear conditioning, without affecting delay conditioning, while the antagonists had no effect. This finding suggests that altered nicotinic signaling in the ventral hippocampus can suppress hippocampus dependent learning. In the mPFC nicotine selectively enhanced trace conditioning though both antagonists also enhanced trace fear conditioning. Unlike in the mPFC or dorsal hippocampus, where nicotine and antagonist induced effects occurred during training, effects in the ventral hippocampus occurred at both training and testing,

suggesting that the ventral hippocampus may be able to modulate acquisition as well as expression of hippocampus dependent learning. Additionally, antagonist infusion into the mPFC during testing produced deficits in expression, suggesting that this area can modulate fear expression. Thus, the substrates of nicotinic acetylcholinergic contributions to trace and contextual fear conditioning are diverse. I put forth a multi-component model of these contributions, where trace fear conditioning is supported by dorsal hippocampus dependent maintenance of the CS during the trace interval, long-term storage in the mPFC and ventral hippocampal mediated acquisition and expression.

Keywords: Nicotine, trace fear conditioning, trace cued fear conditioning, trace-cued fear conditioning, working memory, acetylcholine, dorsal hippocampus, ventral hippocampus, medial prefrontal cortex

Abbreviations: nAChRs nicotinic acetylcholinergic receptors

ACKNOWLEDGMENTS

I would like to acknowledge grant support from the National Institute on Drug Abuse (DA017949 TG), the National Cancer Institute and National Institute on Drug Abuse (P5084718 PI: Caryn Lerman Ph.D). Additionally, I was supported by a NIH/NIDA training grant (T32DA07237).

Additionally, I would like to acknowledge support of other members of the Gould Lab, Justin Kenney, Gabriel Elias & Daniel Gulick without whose support I would not have fully developed the ideas and experiments of which this dissertation is comprised. Also, I would like to acknowledge the guidance of my advisor, Dr. Gould whose tutelage has allowed me to learn the academic and experimental skills necessary for a career in science, as well as my college mentors, Dr. Charles Edwards & Dr. Lisa Unico, who both imparted in me a thirst for understanding behavior & biochemistry, respectively, which has provided me with a unique perspective on psychological questions. Further, I would like to thank members of my core committee, Dr. Kim Curby & Dr. Jason Chein for approving this project and for providing comments that were essential to the composition of this document.

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CHAPTER 1. THE STUDY OF NICOTINE AND COGNITION: A ROLE FOR TRACE FEAR CONDITIONING

Nicotine and Cognition

Smoking is addictive, and highly detrimental to both the individual and society (CDC, 2005; Rice, 1999). Nicotine's actions, mediated through nicotinic acetylcholine receptors, are the primary component of tobacco's addictive liability (Benowitz *et al.*, 1988). Nicotine binds to, activates, and can desensitize nicotinic acetylcholinergic receptors (nAChRs), which are pentameric ligand gated ion channels (Picciotto *et al.*, 2008). Nicotinic acetylcholine receptors in the CNS are composed of multiple subunits (α2-9, β2-4) (Changeux & Taly, 2008). These receptors can be broadly categorized as high-affinity or low-affinity. High-affinity receptors are heteromeric and composed of a combination of α and β subunits, while low-affinity receptors are homomeric and composed of α7 subunits (Luetje *et al.*, 1990). While the rewarding effects of nicotine have a critical role in addiction, nicotine's effects on cognitive processes are also thought to play an important role in addiction (Gutkin *et al.,* 2006; Kenney & Gould, 2008; Watkins *et al.*, 2000) . Nicotine can enhance learning and cognition, as measured by a 5-choice serial reaction time task (Hahn *et al.,* 2003; Semenova *et al.*, 2007), trace fear conditioning (Davis & Gould, 2007; Gould *et al.*, 2004; Raybuck & Gould, 2009), contextual fear conditioning (Gould & Higgins, 2003; Gould & Wehner, 1999; Gould, 2003), for review see (Gould, 2006), and radial arm maze (Brown *et al.,* 2002; Levin *et al.*, 1998; Levin *et al.*, 2004; Levin *et al.*, 2005; Levin *et al.*, 2005), for review see (Levin, 2002), in rodents; and concurrent delayed match to sample performance in

marmosets (Spinelli *et al.*, 2006). Further, In humans similar effects have been demonstrated, acute nicotine enhances performance of the N-Back task, (Ernst *et al.,* 2001; Jacobsen *et al.*, 2004; Jacobsen *et al.*, 2006*;* Kumari *et al.*, 2003; Xu *et al.*, 2006), serial probe recognition (Pineda *et al.*, 1998), prospective working memory (Rusted & Trawley, 2006), visuo-spatial working memory (Dawkins *et al.,* 2007; Smith *et al.*, 2006), although see (Park *et al.*, 2000), and saccade suppression (Rycroft *et al.*, 2006). Effects of nicotine on cognitive processes may contribute to nicotine's addictive liability, acutely by enhancing associative learning thought to support acquisition of addictive behavior (Raybuck & Gould, 2009), and during withdrawal by producing deficits in cognitive abilities, which could debilitate goal directed behavior that supports cessation (Brega *et al.,* 2008; Gutkin *et al.*, 2006; Raybuck & Gould, 2009; Razani *et al.*, 2004). However, while the neural substrates of some of these tasks are understood, nicotine's role in these structures is not well studied. Additionally, while nicotine is shown to affect these forms of cognition, it is not clear whether this suggests that activation of endogenous nicotinic receptors by acetylcholine is important to these processes, or if nicotine is exerting its effects through mechanisms that are not normally involved in these cognitive processes. Trace fear conditioning provides a model of nicotine's effects on cognition with a well-established set of neural substrates in which both the effects of nicotine and the role of endogenous activation of nAChRs can be examined.

Substrates of Trace Fear Conditioning

Trace fear conditioning involves the association of a temporally discontiguous conditioned stimulus (CS) and unconditioned stimulus (US). Thus, in order to associate

the CS and US, a representation of the CS must be maintained during the trace interval. This task depends upon the dorsal hippocampus (Bangasser *et al.,* 2006; Burman *et al.*, 2006; Büchel *et al.*, 1999; Esclassan *et al.*, 2009; Fendt *et al.*, 2005; Knight *et al.*, 2004; McEchron *et al.*, 1998; Quinn *et al.*, 2002; Quinn *et al.*, 2008; Rogers *et al.*, 2006; Trivedi & Coover, 2006; Yoon & Otto, 2007), ventral hippocampus (Burman *et al.,* 2006; Büchel *et al.*, 1999; Czerniawski *et al.*, 2009; Esclassan *et al.*, 2009; Knight *et al.*, 2004; Rogers *et al.*, 2006; Trivedi & Coover, 2006; Yoon & Otto, 2007), and medial prefrontal cortex (Büchel *et al.,* 1999; Knight *et al.*, 2004; McLaughlin *et al.*, 2002; Quinn *et al.*, 2008), however, the role of these areas in trace fear conditioning is not entirely clear. Two theories account for why trace conditioning depends upon these brain regions, as well as how the CS is associated with the US during trace conditioning, contextual bridging and working memory dependence. However, these theories provide different explanations of the cognitive and neural mechanisms that support acquisition of trace fear conditioning.

Contextual bridging accounts for why the dorsal hippocampus is critical to acquisition of trace fear conditioning (Marchand *et al.*, 2004). Contextual bridging is built on the assumption that in fear conditioning the primary role of the hippocampus is the unification of tonic stimuli into a conjunctive contextual representation. Thus, the necessity of the hippocampus for trace fear conditioning would suggest that formation of a unified contextual representation is critical to trace conditioning. Contextual bridging claims that the CS is associated with the context during training, and that the formation of a context-US association allows a link between the CS and US to be formed (Marchand *et al.,* 2003; Marchand *et al.*, 2004; Quinn *et al.*, 2002). Thus, the CS and US are linked

by a secondary association, of which the context is an integral component. In support of this theory, dorsal hippocampal function is critical to both trace and contextual fear conditioning (Bangasser *et al.,* 2006; Burman *et al.*, 2006; Fendt *et al.*, 2005; McEchron *et al.*, 1998; Quinn *et al.*, 2002; Quinn *et al.*, 2008; Rogers *et al.*, 2006; Trivedi & Coover, 2006; Yoon & Otto, 2007). However, it is possible that the role of the hippocampus in trace fear conditioning is due to functions of the hippocampus other than contextual leanring.

A working memory dependent model of trace fear conditioning explains the involvement of multiple neural substrates and cognitive processes in trace fear conditioning. The CS must be maintained during the trace interval in order to be associated with the US. This could be achieved by active maintenance of the CS in working memory (Carter *et al.*, 2003). Much evidence supports the involvement of working memory in trace fear conditioning. For instance, the strength of trace fear conditioning is associated with subjects' awareness of the CS and US (Knight *et al.*, 2006), and conditioning is disrupted by tasks that increase cognitive load (Carter *et al.,* 2003; Han *et al.*, 2003). Further, the brain regions involved in trace fear conditioning are consistent with the involvement of working memory. For instance, in animal models, trace fear conditioning necessitates multiple brain regions associated with completion of working memory dependent tasks; such as the prefrontal cortex, cingulate cortex, perirhinal cortex, and hippocampus (Bang & Brown, 2009; Burman *et al.*, 2006; Chowdhury *et al.*, 2005; Han *et al.*, 2003; Kholodar-Smith *et al.*, 2008; McEchron *et al.*, 1998; Misane *et al.*, 2005; Quinn *et al.*, 2002; Quinn *et al.*, 2005; Quinn *et al.*, 2008; Rogers *et al.*, 2006; Runyan & Dash, 2004; Runyan & Dash, 2005; Weitemier &

Ryabinin, 2004); and in humans trace fear conditioning activates similar substrates (Büchel *et al.,* 1999; Knight *et al.*, 2004). Since the working memory dependent model accounts for the cognitive and neural substrates of trace fear conditioning, it may provide a better explanation of trace fear conditioning, than does contextual bridging.

These theories both explain why the hippocampus would be involved in trace fear conditioning, but the assumptions of contextual bridging suggest that hippocampal involvement in trace and contextual conditioning should not differ. However, immediate early gene activation, a measure of neural activity at the cellular level, shows that following training of trace conditioning different hippocampal subregions are activated than following contextual conditioning (Weitemier & Ryabinin, 2004). Additionally, NMDA receptor antagonism produces deficits in retrieval of trace but not contextual conditioning (Quinn *et al.*, 2005). These findings suggest that the hippocampus is differentially involved in trace and contextual conditioning, but the first of these findings is correlational, and the second involves an effect at testing but not at training, which may not inform how acquisition of the trace-association is supported. The nail in the coffin for contextual bridging would be to demonstrate that hippocampal processes that support acquisition of trace fear conditioning are distinct from hippocampal processes that support acquisition of contextual fear conditioning. To date, no studies have demonstrated this.

Nicotine and Trace Fear Conditioning

Acute nicotine enhances trace fear conditioning, while we do not know where this effect occurs, evidence from contextual fear conditioning, on which nicotine has effects

similar to trace fear conditioning, suggests that the dorsal hippocampus may be involved. The dorsal hippocampus is critical to both trace and contextual fear conditioning (Bangasser *et al.,* 2006; Burman *et al.*, 2006; Fendt *et al.*, 2005; McEchron *et al.*, 1998; Quinn *et al.*, 2002; Quinn *et al.*, 2008; Rogers *et al.*, 2006; Trivedi & Coover, 2006; Yoon & Otto, 2007), and is the critical site for nicotine's effects on contextual fear conditioning (Davis & Gould, 2009; Davis *et al.*, 2007). Thus, it is possible that nicotine's action in the dorsal hippocampus is also critical to nicotine's effect on trace fear conditioning. This would support contextual bridging, as it would demonstrate that similar processes in the dorsal hippocampus modulate both trace and contextual conditioning. Thus, if contextual bridging accurately describes the mechanism that supports trace fear conditioning then nicotine should have identical effects on both trace and contextual conditioning, and activation of nicotinic acetylcholine receptors by endogenous acetylcholine release should have the same role in each of these tasks. However, if working memory dependence explains how the CS and US are associated during trace fear conditioning, then the effects of nicotine in the dorsal hippocampus may be different, and nicotine and activation of nicotinic acetylcholine receptors by acetylcholine may have different effects on trace and contextual fear conditioning in other brain areas involved in trace fear conditioning. By directly comparing the effects of local infusion of nicotine and antagonists for nicotinic acetylcholine receptors in the dorsal hippocampus, ventral hippocampus and medial prefrontal cortex the following series of experiments investigate which theory best explains trace fear conditioning, as well as investigating the local effects of nicotine on trace fear conditioning and the role of nAChRs in trace fear conditioning.

CHAPTER 2. THE EFFECTS OF DORSAL HIPPOCAMPAL NICOTINE ON TRACE FEAR CONDITIONING

Introduction

Though the psychological and theoretical substrates of trace fear conditioning are not fully understood, a number of studies clearly demonstrate that certain brain regions are involved in this task. In addition to the neural substrates involved in delay fear conditioning, such as the amygdala and periaquaductal gray (for review see Fanselow, 1999), trace fear conditioning involves multiple neural substrates associated with higher cognitive functions. Of these substrates, the hippocampus is the most studied. While the hippocampus is organized in terms of multiple regions (CA1, CA3, Dentate Gyrus) and layers (molecular cell layer, granule cell layer, stratum radiatum, stratum oriens, stratum lucidem (Lopes da Silva & Arnolds, 1978), much of the trace fear conditioning literature simply treats the hippocampus as a whole brain region, or sub-divides it into two poles, dorsal and ventral. This generalization may be warranted as direct infusion and lesion studies seldom have the spatial resolution to allow investigation of hippocampal subregions or individual layers, indeed drug diffusion spread is often such that specific targeting of CA1 as opposed to CA3 or the dentate gyrus is problematic. Thus, I will treat the hippocampus as two distinct regions, the dorsal hippocampus and the ventral hippocampus. This chapter reports investigations of the effects of nicotine infusion into the dorsal hippocampus on trace fear conditioning, contextual fear conditioning, and delay fear conditioning.

Addressing the dorsal hippocampus and ventral hippocampus as two distinct regions is supported by more than just the technical limitations inherent in direct drug infusion. Across multiple learning and memory paradigms contributions by the dorsal and ventral hippocampus can be differentiated, with some tasks depending more on one hippocampal pole or the other (Bannerman *et al.,* 2004; Blaker *et al.*, 1984; Czerniawski *et al.*, 2009; Hunsaker & Kesner, 2008; Leonardo *et al.*, 2006; Maggio & Segal, 2007; Maggio & Segal, 2009; Moser & Moser, 1998). Evidence suggests that the dorsal hippocampus plays a critical role in trace fear conditioning.

Converging evidence suggests that the dorsal hippocampus is critically involved in trace fear conditioning, and that this structure may play a specific role in supporting learning and retention of the trace-conditioned CS-US association. Dorsal hippocampal lesions produce deficits in trace fear conditioning (Bangasser *et al.,* 2006; Burman *et al.*, 2006; Fendt *et al.*, 2005; McEchron *et al.*, 1998; Quinn *et al.*, 2002; Quinn *et al.*, 2008; Rogers *et al.*, 2006; Trivedi & Coover, 2006; Yoon & Otto, 2007). Although, it should be noted that the length of trace interval dictates whether the dorsal hippocampus is necessary for acquisition of the CS-US association (Chowdhury *et al.,* 2005; Misane *et al.*, 2005; Quinn *et al.*, 2005 ; Seo *et al.*, 2008; Wanisch *et al.*, 2005). Lesions or NMDA antagonism of the dorsal hippocampus inhibits trace conditioning with a 15-45 second trace interval, but not with shorter intervals, suggesting that unlike trace-eyeblink conditioning where a 500 millisecond trace interval necessitates the hippocampus (Kim *et al.,* 1995; Weiss *et al.*, 1999), in trace fear conditioning longer trace intervals are necessary to for hippocampal dependence. Additionally trace fear conditioning activates intracellular signaling cascades, such as ERK in the dorsal hippocampus (Runyan &

Dash, 2004; Villarreal & Barea-Rodriguez, 2006), an event which may be critical to learning since systemic ERK inhibition produces deficits in trace fear conditioning (Villarreal & Barea-Rodriguez, 2006). Further, immediate early gene expression in the dorsal hippocampus increases following trace fear conditioning (Weitemier & Ryabinin, 2004). In rats CA1 and DG neurons show distinct patterns of activity during CS presentations across the trace conditioning session, with CA1 decreasing in response to the CS and DG increasing in response to the CS (Gilmartin & McEchron, 2005). Collectively these studies demonstrate a critical role for the dorsal hippocampus in trace fear conditioning. However, it is not clear whether nicotinic acetylcholinergic signaling in the dorsal hippocampus is involved in trace fear conditioning.

Scientific Questions: (Design)

These studies investigated the effects of dorsal hippocampal nicotine infusion on trace fear conditioning. Initially I generated a dose response curve to determine the optimal dose of nicotine in the dorsal hippocampus for producing effects on trace fear conditioning. Subsequently I used the optimal dose (0.09 μg/side) to determine if nicotine was diffusing above or below the dorsal hippocampus to exert its effects. Additionally, to determine if nicotine's effects on the contextual component of trace conditioning were particular to a 5-pairing training protocol, I trained mice treated with high or low doses of nicotine with 2-pairing trace fear conditioning. Then, to confirm that nicotine was not simply affecting cued learning we infused nicotine into the dorsal hippocampus prior to delay fear conditioning (i.e. CS-US training with no trace interval), with two different training protocols, to rule out the possibility of ceiling effects. Finally,

we infused nicotine prior to either training or testing of trace fear conditioning to determine if nicotine was enhancing processes related to acquisition or processes related to expression of trace fear conditioning.

Methods

Subjects

These experiments used 193 8-12 week old male C57BL/6J mice. All mice were singly housed in standard colony cages, maintained on a 12h light/dark cycle with lights on at 7:00 am, and allowed *ad libitum* access to food and water. Housing, surgical, and behavioral procedures were approved by the Temple University Animal Care and Use Committee, and were in accordance with APA ethical standards.

Materials

Drugs and Infusion

Nicotine hydrogen tartrate salt [reported as freebase] 0.045, 0.09, 0.18, & 0.35 μg/side was obtained from Sigma-Aldrich (St. Louis, MO). Nicotine was directly infused through 22-gauge cannula. During infusions mice were gently restrained; then stainless steel stylets were removed from guide cannula and replaced with infusion cannula. Drugs were infused at a rate of 0.50 μl/min and at an injection volume of 0.50 μl per side. Infusion cannula were attached to polyethylene tubing (PE50; Plastics One) attached to a 10 μl Hamilton syringe (Reno, NV), which was controlled by a microinfusion pump

(KDS 100; KD Scientific, New Hope, PA). Injection cannula were left in place for 30 seconds after infusion. Nicotine was infused immediately before training and/or testing. Spread of infusion using this procedure has been previously estimated to be ~1mm^3 (Lewis & Gould, 2007).

Apparatus

Training was conducted in conditioning chambers (model 307AW, Med Associates, St. Albans, VT) housed in sound attenuating cubicles. An 85 dB white noise CS was administered through speakers attached to the right wall of each chamber. A 2 second, 0.57 mA US was administered with a shock generator and scrambler (Med-Associates) through the chamber floors, which were composed of 18 stainless steel bars connected to a shock generator and scrambles. 69 dB background noise was provided by 50 mm ventilation fans, mounted on the right wall of each sound-attenuating cubicle. Stimulus administration was controlled by an IBM-compatible PC running Med-PC software (Med-Associates).

Altered and cued fear conditioning was tested in four conditioning chambers situated in sound attenuating cubicles located in a different room than that used for training. The testing chambers were distinct from the training chambers and had white plastic floors, stainless steel sides, and Plexiglas panels for the front, rear and lid. Additionally, a novel olfactory cue (artificial vanilla extract) was applied to paper toweling placed below each of the chamber floors. Ventilation fans mounted on the right wall of the sound attenuating cubicles provided background noise. CS was generated

with a Grason-Stradler noise generator (model 901B, West Concord, MA) attached to 3-inch speakers mounted on the left side of each of the conditioning chambers.

Procedure

Surgical

For surgical procedures mice were anesthetized with isoflurane, and placed in a mouse stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). The scalp was shaved, scrubbed and retracted to expose the skull. Holes were drilled for anterior and lateral coordinates, determined from Paxinos and Franklin (2001) with respect to bregma. Guide cannula were lowered into place with the stereotaxic, to match D/V coordinates, and permanently secured with dental cement. Dorsal hippocampal coordinates were A/P – 1.7, M/L 1.5, D/V 2.3; above D/V 1.5, below D/V 2.3. All cannula were purchased from Plastics One (Roanoke, Virginia). Stainless steel stylets were placed in the guide cannula to maintain patency during the minimal 5-day recover period. All animals received postoperative analgesic/anti-inflammatory ketprofen (2 mg/kg, sc; Fort Dodge, Fort Dodge, IA).

Behavioral

Trace Fear Conditioning: Behavioral procedures were based on previous studies (Davis & Gould, 2007; Gould *et al.*, 2004). Training of trace fear conditioning was conducted during a single 15 minute and 30 second training session wherein the mice were presented 5 CS-US pairings separated by a variable inter-trial-interval (90-120 seconds). CS-US parings consisted of a 30 second, 85 dB white noise CS presentation, followed by

a 30 second trace interval, and terminated with the presentation of a 2 second, 0.57 mA footshock US. The training session began with activation of the house light and terminated 30 seconds following the last US presentation, at which point the house light was extinguished and mice were placed in their home cage. Twenty-four hours following training, mice were tested for both contextual and trace-cued associative conditioning. To test for contextual associations formed during the training session mice were placed in the training context and observed for freezing for five minutes. One to two hours later, mice were placed in the altered testing chambers for six minutes to test for trace-cued conditioning; for the first three minutes no CS was presented and mice were scored for freezing to the altered context, a measure of generalized fear, then the CS was presented for three minutes and mice were scored for trace cued freezing.

In addition to 5-pairing trace fear conditioning, a subset of studies used a 2-pairing trace fear conditioning protocol where mice received two CS-US pairings with a 30 second trace-interval and a 90 second inter-trial interval. These training sessions lasted 6 minutes and 30 seconds. All other parameters were as described previously.

Delay Fear Conditioning: Delay fear conditioning procedures are based on previous studies (Davis *et al.,* 2007; Gould & Higgins, 2003; Gould, 2003). Training of delay fear conditioning was conducted during a single 5 minute and 30 second training session wherein the mice were presented with 2 CS-US pairings separated by a 90 second inter-trial-interval. CS-US parings consisted of a 30 second 85 dB white noise CS presentation, which co-terminated with a 2 second 0.57 mA footshock US. The training session began with the activation of the house light and terminated 30 seconds following

the last US presentation, at which point the house light was extinguished and mice were placed in their home cage. Testing of both contextual and delay-cued conditioning was performed as described above. In addition to the 2-pairing delay conditioning training protocols, a subset of experiments utilized a 1-pairing delay fear conditioning paradigm, in which mice received only one CS-US pairing, and the CS was only 15 seconds in duration. Otherwise, training and testing parameters for both delay conditioning protocols were identical.

Histology

All brains were post-fixed in formalin for at least 24 hours, sliced on a cryostat, mounted, and nissl stained. Infusion coordinates were confirmed with either dye infusion, or by observing gliosis along the infusion cannula tracts. Cannula placements outside of the target area (<2%) were excluded from analysis.

Analysis

Analysis was conducted with SPSS 17. Data were analyzed with one-way ANOVA, followed with Tukey's HSD post hoc tests or with an independent-samples t-test. Data sets not meeting homogeneity of variance assumption of the ANOVA were followed up with a Games-Howell post-hoc test.

Results

Placements

Histological analysis of all dorsal hippocampal nicotine infusions showed that out of 193 surgical cannula implantations 3 were outside of the target area. Placements that did not hit the target area were excluded from analysis. Diagrams of placements for each study are displayed with the behavioral data.

Effects of dorsal hippocampal nicotine infusion on trace fear conditioning.

Multiple studies suggest that the dorsal hippocampus is a critical structure in the acquisition of trace conditioning and that nicotine can act within the dorsal hippocampus to alter learning (Davis & Gould, 2009; Davis *et al.*, 2007; Esclassan *et al.*, 2009; Quinn *et al.*, 2008). Thus, to determine if the dorsal hippocampus is involved in the effects of nicotine on trace fear conditioning, I infused nicotine at a range of doses into this structure prior to training and testing of trace fear conditioning. ANOVA showed that infusion of nicotine into the dorsal hippocampus affected both contextual conditioning [$F(4,61) = 3.809$, $p < 0.008$] and cued conditioning [$F(4,61) = 4.683$, $p < 0.002$], but had no effect on baseline or altered freezing, Figure 1. Post hoc analysis indicated that mice receiving 0.09 μg/side of nicotine froze more to the context than saline treated mice and that mice receiving 0.09 or 0.18 μg/side froze more to the cue than controls.

Figure 1.

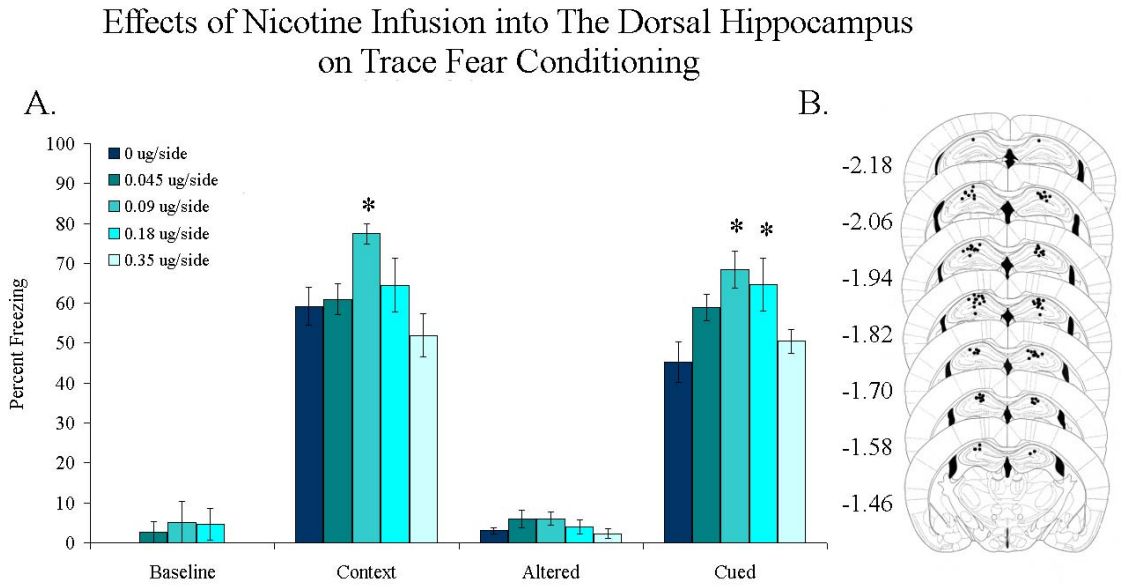

Figure 1: Dorsal hippocampal nicotine infusion dose-dependently enhances trace and contextual far conditioning. A. Infusion of nicotine (0.09 µg/side contextual, 0.09 & 0.18 µg/side cued) prior to trace fear conditioning enhanced contextual and cued components, but had no effect on baseline or altered freezing. Significant difference ($p < 0.05$) from saline treated control group denoted with (*) data are reported as mean ± standard error of the mean. Subjects per group were 13 (0.0, 0.45, 0.09 µg), 11 (0.18 µg), and 12 (0.35 µg). B. Subsequent histological analysis confirmed that all but 3 placements were within the dorsal hippocampus.

Diffusion controls for the effects of dorsal hippocampal nicotine on trace fear conditioning.

Above the dorsal hippocampus

Infusion of nicotine into the dorsal hippocampus produced enhancement of trace and contextual fear conditioning, however, it is possible that nicotine's effects were due to diffusion up the cannula tract into cortical areas lying directly above the dorsal hippocampus. To determine if apparent effects of dorsal hippocampal nicotine infusion on trace fear conditioning were due to nicotine diffusion up the cannula tract, I infused an

effective dose of nicotine (0.09 µg/side) above the dorsal hippocampus prior to training and testing of trace fear conditioning. T-tests showed that infusion of 0.09 µg/side nicotine above the dorsal hippocampus had no effect on baseline, contextual, altered, or cued freezing, Figure 2.

Figure 2.

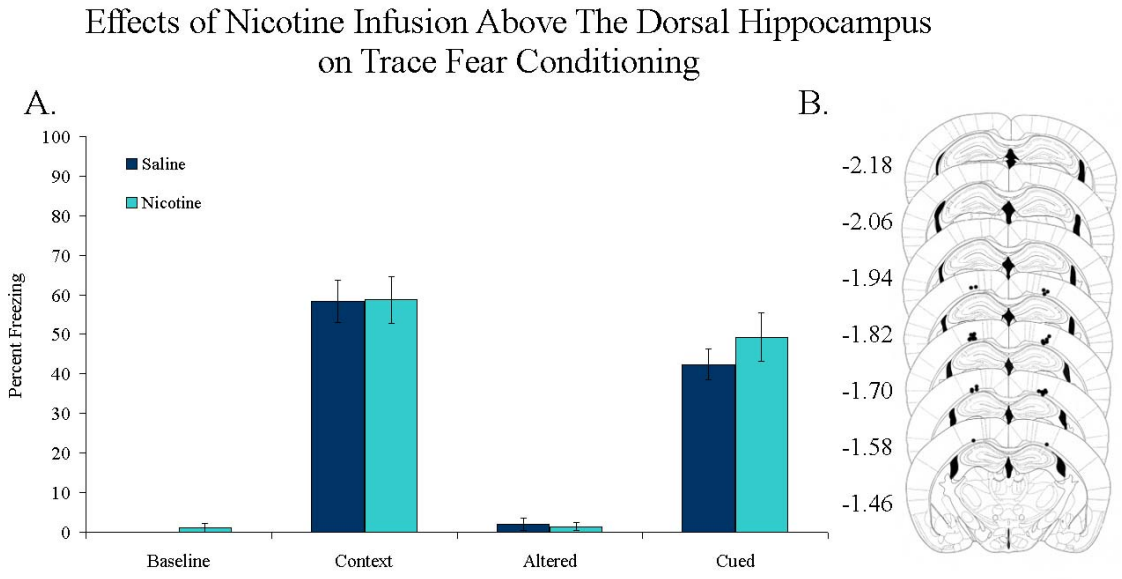

Figure 2: Infusion of nicotine above the dorsal hippocampus had no effect on trace fear conditioning. A. **Infusion of nicotine (0.09 µg/side) directly above the dorsal hippocampus had no effect on any measures of trace conditioning. There were 8 subjects per group.** B. **Histological placements confirmed that all placements were in cortical areas directly above the dorsal hippocampus.**

Below the dorsal hippocampus

Infusion of nicotine into the dorsal hippocampus enhanced trace fear conditioning, while this effect was not due to nicotine diffusing up the cannula tract, it is possible that this effect is due to nicotine diffusing out of the dorsal hippocampus into

dorsal thalamic nuclei. To determine if effects of dorsal hippocampal nicotine infusion on trace fear conditioning were due to nicotine diffusing through the hippocampus into dorsal thalamic nuclei, I infused an effective dose of nicotine (0.09 µg/side) below the dorsal hippocampus prior to training and testing of trace fear conditioning. T-tests showed that infusion of 0.09 µg/side nicotine below the dorsal hippocampus had no effect on baseline, contextual, altered, or cued freezing, Figure 3.

Figure 3.

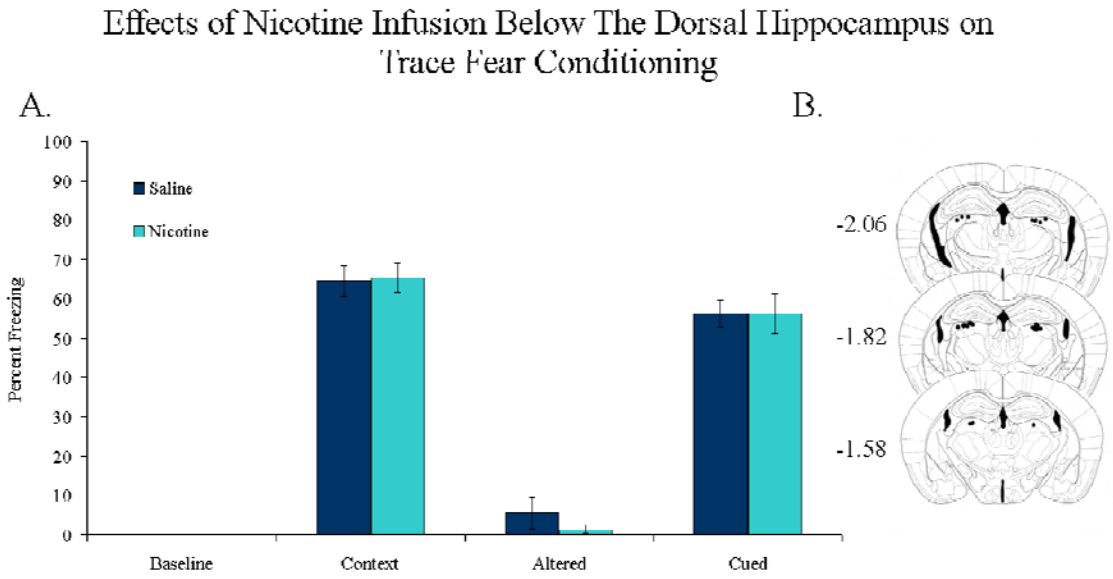

Figure 3: Infusion of nicotine below the hippocampus into the dorsal thalamus had no effect on trace fear conditioning. A. **Nicotine infusion (0.09 µg/side) into the dorsal thalamus had no effect on trace fear conditioning. There were 8 subjects per group.** B. **Histological analysis verified that all placements were in the dorsal thalamus directly below the dorsal hippocampus.**

Effects of dorsal hippocampal nicotine infusion on 2-pairing trace fear conditioning.

The previous findings show that dorsal hippocampal nicotine infusion enhances both trace and contextual fear conditioning, but most contextual data involves training with 2 CS-US pairings, not the 5 pairings used for trace conditioning. Thus, to examine if nicotine's effects in a 5-pairing trace conditioning paradigm are the result of this particular training paradigm, I used a 2-pairing trace conditioning paradigm, to determine if the enhancement of trace fear conditioning by dorsal hippocampal nicotine infusion is specific to trace conditioning, and not due to extent of training. I infused nicotine at 0.09 or 0.35 μg/side into the dorsal hippocampus prior to training and testing of a 2-pairing trace fear conditioning paradigm. ANOVA showed that infusion of nicotine into the dorsal hippocampus affected contextual [$F(2,21) = 10.467$, $p < 0.001$] and cued [$F(2,21) = 10.642$, $p < 0.001$] conditioning, but did not affect baseline or altered freezing. Post hoc analysis revealed that the higher dose of nicotine (0.35 μg/side) enhanced contextual conditioning, and that the lower dose of nicotine enhanced cued conditioning. Thus, the effects of dorsal hippocampal nicotine infusion on contextual and trace conditioning are dissociable by dose.

Figure 4.

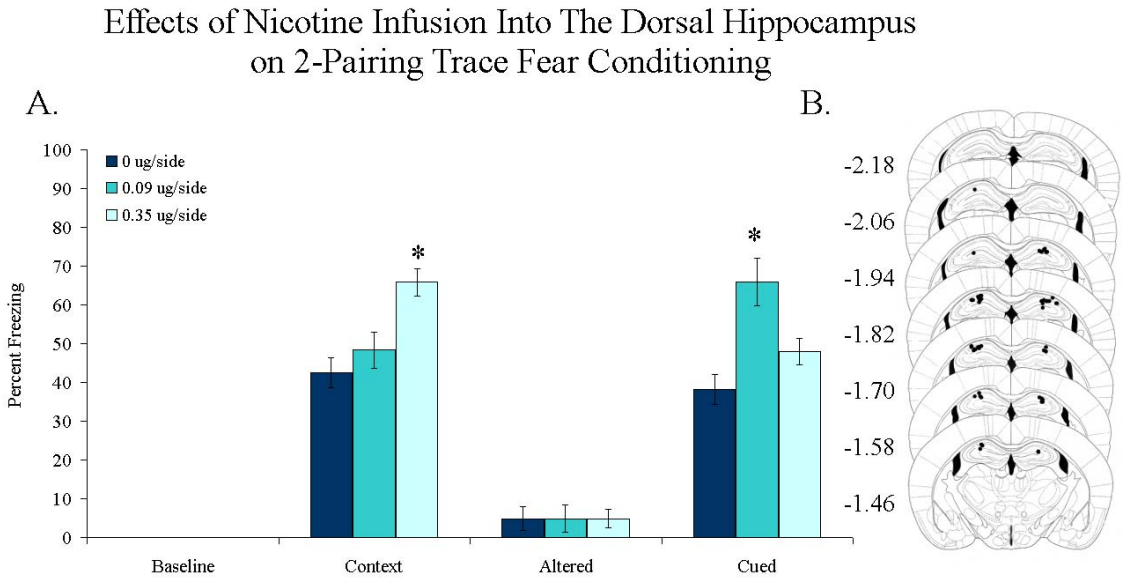

Figure 4: Infusion of nicotine into the dorsal hippocampus enhances trace fear conditioning regardless of training protocol. A. **Nicotine infusion at 0.09 µg/side into the dorsal hippocampus enhanced trace fear conditioning, but not contextual conditioning, while nicotine infusion at 0.35 µg/side enhanced contextual but not trace conditioning, in a 2-pairing trace fear conditioning protocol. Significant difference ($p < 0.05$) from saline treated control group denoted with (*) data are reported as mean ± standard error of the mean. There were 8 subjects per group.** B. **Histological analysis confirmed that all drug infusions were directed into the dorsal hippocampus.**

Effects of dorsal hippocampal nicotine infusion on delay fear conditioning

2-pairing delay fear conditioning

Infusion of nicotine into the dorsal hippocampus enhances trace fear conditioning, however it is possible that this effect may be driven by an effect of nicotine on stimulus processing that is not limited to trace conditioning. Thus, it may be the case that nicotine infusion into the dorsal hippocampus enhances stimulus saliency resulting in enhancement of cued conditioning in general. To determine if effects of dorsal hippocampal nicotine infusion on trace conditioning were due to non-specific

enhancement of cued-conditioning, I infused nicotine at 0.09 and 0.35 µg/side into the dorsal hippocampal prior to training and testing of delay fear conditioning (i.e. no trace interval). ANOVA showed that infusion of nicotine into the dorsal hippocampus affected contextual conditioning [$F(2,21) = 9.596$, $p < 0.001$], but not baseline, altered, or cued freezing, Figure 5. Post hoc analysis demonstrated that mice treated with 0.35 µg/side nicotine froze more to the context than mice treated with saline, or with 0.09 µg/side nicotine.

Figure 5.

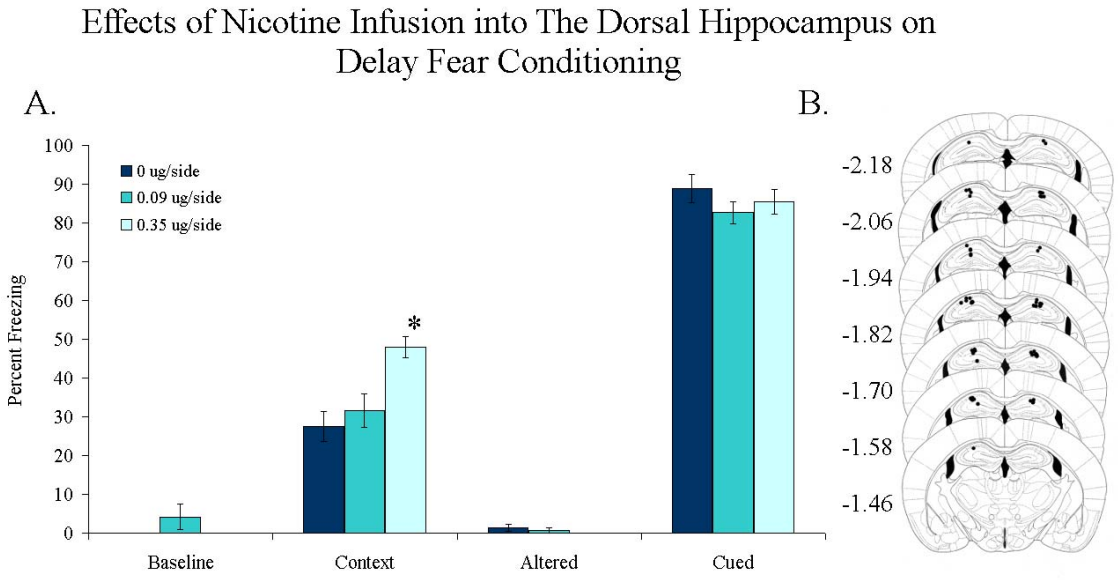

Figure 5: Infusion of nicotine into the dorsal hippocampus enhances contextual but not delay fear conditioning. A. **Infusion of nicotine (0.35 µg/side) into the dorsal hippocampus enhanced the contextual component of a 2-pairing (30s CS) delay fear conditioning but infusion (0.09 & 0.35 µg/side) had no effect on delay conditioning. Significant difference ($p < 0.05$) from saline treated control group denoted with (*) data are reported as mean ± standard error of the mean. There were 8 subjects per group.** B. **Histological analysis confirmed that all infusions were directed into the dorsal hippocampus.**

1-pairing delay fear conditioning

Infusion of nicotine into the dorsal hippocampus (0.35 μg/side) enhances contextual conditioning but has no effect on delay fear conditioning. However, it is possible that effects of nicotine on delay fear conditioning could be masked by high freezing levels of mice conditioned with a 2-pairing, 30s CS delay conditioning protocol. This protocol has been shown to produce lower levels of freezing, preventing a ceiling effect from obscuring possible drug effects (Davis *et al.,* 2007; Gould *et al.*, 2004), and while delay conditioning using this protocol is not enhanced by dorsal hippocampal nicotine infusion at 0.35 μg/side, the effects of a lower dose have not been investigated. Thus, I used a less intensive conditioning protocol with 1 CS-US pairing and a 15s CS duration to evaluate the effects of 0.09 μg/side nicotine infusion into the dorsal hippocampus prior to training and testing. T-tests showed that infusion of 0.09 μg/side nicotine prior to 1-pairing delay conditioning into the dorsal hippocampus had no effect on baseline, contextual, altered, or cued freezing as compared to saline (0 μg/side) receiving controls, Figure 6.

Figure 6.

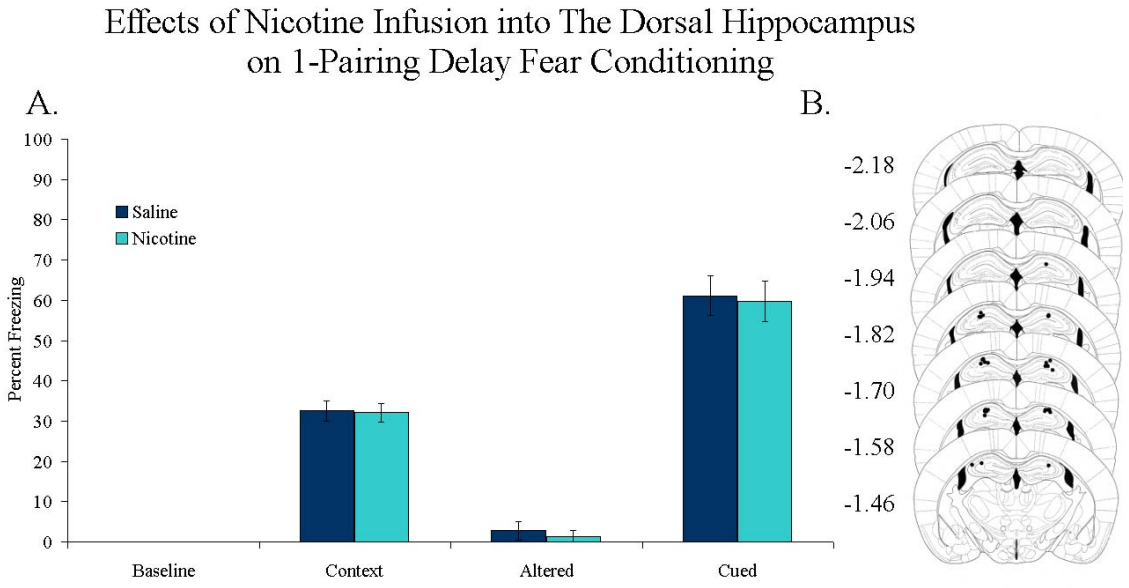

Figure 6: Nicotine infusion into the dorsal hippocampus does not affect delay fear conditioning. A. **Dorsal hippocampal nicotine infusion (0.09 µg/side) had no effect on contextual or cued measures of delay fear conditioning in a 1-pairing 15s CS conditioning protocol. There were 8 subjects per group.** B. **Histological analysis confirmed that drug infusions were directed at the dorsal hippocampus.**

Effects of dorsal hippocampal nicotine infusion at training or testing of trace fear conditioning.

Infusion of nicotine into the dorsal hippocampus selectively enhances trace and contextual fear conditioning and these effects are dissociable by dose, but it is not clear that these effects occur through modulation of processes engaged during acquisition or during retrieval of trace and contextual fear conditioning. Evidence from the literature suggest that the dorsal hippocampus is involved in both acquisition and retrieval of trace fear conditioning, but that with older memories the dorsal hippocampus is not involved retrieval of trace conditioning (Quinn *et al.*, 2008). However, until now no study has reported whether the enhancing effects of nicotine on trace or contextual fear

conditioning occur at training or at testing, although recent work suggest that nicotine's effects are on learning of the context but not learning of the context-shock association (Kenney & Gould, 2008), and no studies have reported effects of nicotine in the dorsal hippocampus at training or testing of fear conditioning. Thus, to determine whether nicotine infusion into the dorsal hippocampus alters acquisition or expression of trace fear conditioning, I infused an effective (0.09 u/side) dose of nicotine at either training or testing, or on both days of trace fear conditioning (5 CS-US pairings). ANOVA showed that infusion of nicotine affected contextual [$F(2,21) = 10.642$, $p < 0.001$] and cued [$F(3,28) = 9.579$, $p < 0.000$] conditioning, but not baseline or altered freezing, Figure 7. Post hoc analysis revealed that nicotine infusion at training or at both training and testing enhanced contextual and cued conditioning and that infusion of nicotine at testing had no effect.

Figure 7.

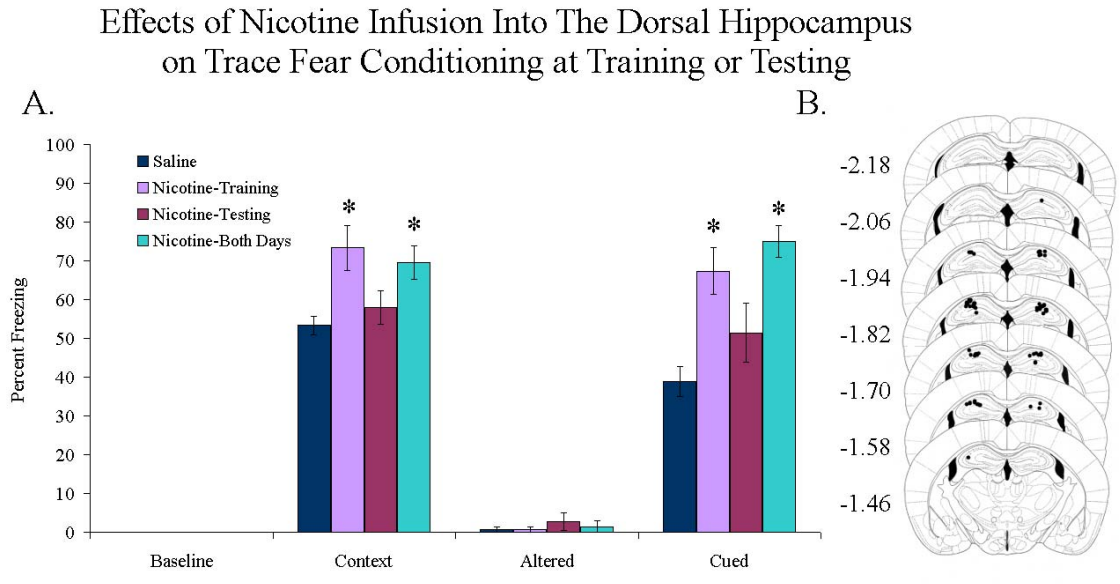

Figure 7: Enhancement of trace and contextual fear conditioning by dorsal hippocampal nicotine infusion occurs at training but not at testing. A. **Infusion of 0.09 µg/side nicotine into the dorsal hippocampus at training or at training and testing of trace fear conditioning was sufficient to enhance both contextual and cued components of this task. However, infusion at testing had no effects. Thus, nicotine's effects on trace and contextual fear conditioning in the dorsal hippocampus occur at training but not at testing. Significant difference ($p < 0.05$) from saline treated control group denoted with (*) data are reported as mean ± standard error of the mean. There were 8 subjects per group.** B. **Histological analysis confirmed that drug infusions were directed at the dorsal hippocampus.**

Conclusions

These studies demonstrate that nicotine infusion into the dorsal hippocampus enhances acquisition of both trace and contextual fear conditioning, and demonstrate that in a 2-pairing trace fear conditioning paradigm these two effects occur at different doses. Additionally, these studies demonstrate that nicotine infusion above or below the dorsal hippocampus has no effects on trace fear conditioning, suggesting that nicotine's action on this task is occurring within the dorsal hippocampus. Further, these studies show that nicotine infusion into the dorsal hippocampus has no effect on delay fear conditioning.

Collectively, these finding suggest that the enhancing effects of systemic nicotine on trace fear conditioning could be mediated by nicotine's action in the dorsal hippocampus.

Dorsal hippocampal nicotine enhances trace fear conditioning in an inverted u-shaped dose response curve

The dose-response effects of dorsal hippocampal nicotine fit with the effects of systemic nicotine on hippocampus dependent contextual fear conditioning. Enhancement of trace fear conditioning was significant at doses of 0.09 and 0.18 μg/side, but not at lower or higher doses, this range was also matched by in 2-pairing trace conditioning, with enhancement at 0.09 μg/side but not 0.35 μg/side. This effect is consistent with systemic effects of nicotine on contextual conditioning where enhancement is also seen in an inverted u-shaped dose response curve (Gould & Higgins, 2003). It is not yet known if enhancement of trace fear conditioning by systemic nicotine also occurs in this manner. McGaugh suggests that memory enhancing drugs should typically exert effects in an inverted u-shaped curve, (McGaugh & Roozendaal, 2009), because the systems affected by these drugs can have detrimental effects on learning when over activated. While the higher dose did not produce deficits in conditioning, it is unclear if doses higher that those tested here would produce deficits in trace conditioning. Additionally, it is possible that nicotine is enhancing trace conditioning through a mechanism that is not normally involved in this task, if this were the case then dorsal hippocampal infusion of nicotinic antagonists would be expected to have no effect on trace conditioning. This hypothesis will be investigated in Chapter 5.

Dorsal hippocampal nicotine has different effects on trace and contextual fear conditioning

The present findings demonstrate that dorsal hippocampal nicotine has similar dose effects on both trace and contextual fear conditioning following training with 5 CS-US pairings, but with 2 CS-US pairings dorsal hippocampal nicotine has different dose effects on trace versus contextual fear conditioning. Comparison of 5-pairing and 2-pairing trace conditioning shows that nicotine's effects on the contextual component of trace conditioning increases in sensitivity with more CS-US pairings, while nicotine's effects on trace conditioning occur at the same dose, regardless of CS-US pairings. Thus, it seems likely that these effects of nicotine in the dorsal hippocampus on trace and contextual fear conditioning are supported by different mechanisms.

NMDA receptor activation in the dorsal hippocampus and subsequent activation of extra-cellular regulated protein kinase 1/2 (ERK 1/2) are critical to contextual fear conditioning (Atkins *et al.,* 1998; Bast *et al.*, 2003; Quinn *et al.*, 2005; Trifilieff *et al.*, 2006). Additionally, subthreshold inhibition of NMDA receptor or ERK 1/2 activation blocks enhancement of contextual fear conditioning by nicotine (Gould & Lewis, 2005; Raybuck & Gould, 2007). The shift in sensitivity to enhancement of contextual fear conditioning by dorsal hippocampal nicotine administration may be because more US presentations result in more activation of NMDA and ERK 1/2, thus with more presentations less activation of nAChRs is necessary to produce enhancement. However, in trace fear conditioning, the same dose of dorsal hippocampal nicotine enhances conditioning, regardless of training protocol. This suggests that nicotine's effects in the dorsal hippocampus on trace fear conditioning are mediated by a different mechanism

than nicotine's effects on contextual conditioning. For instance, it may be that during contextual learning nicotine is enhancing plasticity related events, which increase with more training, while during trace fear conditioning nicotine is acting in the dorsal hippocampus to enhance maintenance of the CS, which could be the same process regardless of the number of CS-US pairings, the implications of these findings are discussed in Chapter 6.

Dorsal hippocampal nicotine infusion enhances acquisition of trace and contextual fear conditioning

The present findings demonstrate that in the dorsal hippocampus nicotine acts during training to enhance acquisition of both trace and contextual fear conditioning. While previous studies have demonstrated that precipitated nicotine withdrawal deficits occur at training of trace and contextual fear conditioning (Portugal *et al.,* 2008; Raybuck & Gould, 2009), and that infusion of nicotine into the dorsal hippocampus at both training and testing enhances contextual conditioning (Davis *et al.*, 2007), this is the first study to demonstrate that nicotine's action on trace or contextual conditioning occurs during training. This finding is significant because previous studies have shown that systemic nicotine is necessary at both training and testing for enhancement to occur (Gould & Higgins, 2003), although nicotine is no longer necessary for enhancement one week after training. The present findings show that in the dorsal hippocampus, where activation of high-affinity nicotinic receptors is critical to the enhancement of contextual fear conditioning by systemic nicotine (Davis *et al.*, 2007), nicotine's effects on both contextual and trace fear conditioning occur at training, suggesting that brain regions

other than the dorsal hippocampus may be mediating the necessity of systemic nicotine at both training and testing of contextual fear conditioning.

Nicotine is not affecting trace or contextual conditioning by diffusing into areas adjacent to the dorsal hippocampus

In any infusion study it is important to demonstrate that drug is reaching the target brain area, and to demonstrate that effects are not due to drug diffusing into a brain area adjacent to the target or diffusing throughout the brain. Nicotine is a difficult drug to infuse because it is highly amphipathic, meaning that it will diffuse freely throughout both lipid and aqueous tissue, e.g. cell membranes and extra-cellular fluid, and may not be limited to the brain area into which it was infused. However, it is possible to demonstrate regional specificity by showing that nicotine is not acting on adjacent areas to exert its effects and that nicotine is not exerting its effects by spreading throughout the entire brain. Effects of dorsal hippocampal nicotine infusion cannot be attributed to diffusion of nicotine up the cannula tract or to diffusion of nicotine downward into the dorsal thalamus, as infusion either above or below the dorsal hippocampus had no effect on any measures of fear conditioning.

Dorsal hippocampal nicotine infusion enhances trace and contextual conditioning but not delay conditioning

Dorsal hippocampal nicotine infusion (0.35 μg/side) enhanced contextual conditioning without affecting delay conditioning. These findings are consistent with the systemic effects of nicotine on trace fear conditioning where systemic administration of

0.09 mg/kg nicotine enhanced trace fear conditioning and contextual fear conditioning, without affecting delay conditioning (Gould & Higgins, 2003; Gould & Wehner, 1999; Gould *et al.*, 2004). Thus, nicotine's effects in the dorsal hippocampus on trace and contextual fear conditioning are not due to alteration of anxiety or locomotion, but rather reflect enhancement of learning of the trace conditioned CS-US association and learning of the context-US association, because changes in anxiety or locomotion would also affect delay fear conditioning.

Summary

Collectively the findings from this chapter demonstrate that dorsal hippocampal nicotine infusion enhances acquisition of trace and contextual fear conditioning in a dose responsive manner with an inverted u-shaped dose curve, that the effects of dorsal hippocampal nicotine on trace and contextual fear conditioning are mediated by different mechanisms, and that these effects are not due to diffusion of nicotine to areas adjacent to the dorsal hippocampus or due to effects of infusion on locomotion or anxiety. The most important of these findings is that nicotine's effects on trace and contextual fear conditioning are dissociable, this finding has implications for the role of the dorsal hippocampus in trace fear conditioning and for theories of trace fear conditioning, both of which will be discussed in Chapter 6.

CHAPTER 3. THE EFFECTS OF VENTRAL HIPPOCAMPAL NICOTINE ON TRACE FEAR CONDITIONING

Introduction

Investigations reported in Chapter 2 demonstrate that nicotine's action in the dorsal hippocampus can enhance both trace and contextual fear conditioning, however, this effect does not rule out the possibility that other brain areas are involved in nicotine's effects on trace fear conditioning. One other brain area implicated in trace fear conditioning is the ventral hippocampus. This chapter reports investigations of the effects of nicotine in the ventral hippocampus on trace fear conditioning.

A number of studies suggest that the ventral hippocampus is critical for trace fear conditioning. However, the specific methods and controls used in these studies renders their conclusions less than clear. Trivedi and colleagues (2006) used NMDA to lesion the dorsal or ventral hippocampus in rats and found that lesions to either structure produced deficits in trace conditioned fear potentiated startle. In trace fear conditioning, Rogers and colleagues (2006) used ibotenic acid to lesion the CA1 subregion of the rat dorsal or ventral hippocampus. They found that ventral lesions had no effect on freezing during training, but that during testing of context, CS and post-CS freezing, ventral CA1 lesioned animals froze less than dorsal CA1 lesioned animals, and that both lesion groups froze less than sham operated controls. From this they concluded that hippocampal involvement in trace fear conditioning is graded along the temporal axis, with the ventral hippocampus being more involved than the dorsal hippocampus and that the ventral hippocampus is critically involved in retention of trace conditioning. One potential

caveat of this study is that the authors used a 10 second trace interval. Studies suggest that hippocampal involvement in trace fear conditioning is temporally graded (Chowdhury *et al.,* 2005; Misane *et al.*, 2005), thus hippocampal lesioned animals can acquire CS-US association if the CS and US are separated by a short trace-interval (1-10 seconds) but not if the CS and US are separated by a long trace interval (15-45 seconds). A 10 second trace interval may not require hippocampal involvement. Thus, it is possible that lesions to the dorsal and ventral CA1 produced deficits in expression of conditioned fear that are not specific to trace fear conditioning. However, other researchers have used lesion techniques to investigate the role of the ventral hippocampus in trace fear conditioning with a more appropriate 30 second trace interval; Yoon and Otto (2007) used NMDA to lesion the dorsal or ventral hippocampus of rats prior to or after training of trace fear conditioning. They found that pre-training lesions to the ventral hippocampus produced deficits in acquisition and retrieval of trace fear conditioning, while pre-training dorsal lesions had no effect, and that both post-training ventral and dorsal lesions produced deficits in retrieval of trace fear conditioning. Both Yoon and Otto (2007) and Rogers and colleagues (2006) used extensive training session with 10 and 15 CS-US presentations, respectively. Since studies demonstrating that trace fear conditioning depends on the dorsal hippocampus have used 8, 5, 2, and 1 CS-US pairings (Chowdhury *et al.,* 2005; Misane *et al.*, 2005; Quinn *et al.*, 2005; Quinn *et al.*, 2008; Runyan & Dash, 2005), it is possible that training with 10 or 15 pairings reduces hippocampus dependence. Additionally, neither of these studies used a standard delay conditioned control group to demonstrate that ventral lesions did not produce deficits in expression of delay conditioned fear response, which is hippocampus independent

(Phillips & LeDoux, 1992). Thus, it is impossible to conclude that lesions to the ventral hippocampus had effects specific to trace conditioning. These studies suggest that the ventral hippocampus is critical to trace fear conditioning, however, use of insufficient trace interval and lack of delay fear conditioned controls leaves the possibility that the ventral hippocampus could play a less specific role in trace fear conditioning.

Studies investigating the role of the ventral hippocampus in trace fear conditioning that included appropriate control groups suggest that the ventral hippocampus is involved not just in trace fear conditioning but in delay fear conditioning as well. Esclassan and colleagues (2008) used a combination of whole hippocampal lesions and temporary inactivation to investigate the role of the dorsal and ventral hippocampal poles in trace, contextual, and delay fear conditioning. They found that whole hippocampal lesions produced deficits not only in contextual and trace fear conditioning, but also in delay fear conditioning. They followed this with inactivation of the dorsal or ventral hippocampus with muscimol. Inactivation of the ventral hippocampus had effects similar to whole hippocampal lesion, producing deficits in trace, contextual and delay fear conditioning. Inactivation of the dorsal hippocampus on the other hand selectively produced deficits in trace and contextual fear conditioning, but did not affect delay conditioning. These findings are supported by those of Burman and colleagues (2006) who used trace conditioned potentiation of startle response in conjunction with electrolytic lesions to demonstrate that the dorsal hippocampus is necessary for acquisition of trace conditioning, while the ventral hippocampus is necessary for retrieval of both trace and delay conditioning. Further support of a critical role for the dorsal hippocampus in trace conditioning comes from trace eye-blink

conditioning, where single unit activity during the trace interval was greater in the dorsal hippocampus than in the ventral hippocampus (Weible *et al.*, 2006). Multiple studies report a role for ventral hippocampus in both contextual and delay-cued fear conditioning (Anagnostaras *et al.,* 2001; Bast *et al.*, 2001; Clark *et al.*, 1992; Maren, 1999). Collectively these studies suggest that while the ventral hippocampus is involved in expression of fear response, the dorsal hippocampus is specifically involved in trace and contextual fear conditioning. Thus, a compound like nicotine, which selectively enhances trace and contextual fear conditioning (Gould & Wehner, 1999; Gould *et al.*, 2004), but does not affect delay conditioning, would be expected to act in the dorsal but not the ventral hippocampus.

Studies using lesion techniques and temporary inactivation suggest that the ventral hippocampus is involved in fear conditioning, but the role of nAChRs in these effects are unknown. Although, other studies have shown a critical role for nicotinic acetylcholinergic signaling in the ventral hippocampus in spatial learning (Arthur & Levin, 2002; Bancroft & Levin, 2000; Bettany & Levin, 2001; Kim & Levin, 1996; Levin *et al.*, 1999; Levin *et al.*, 2003; Pocivavsek *et al.*, 2006). Thus, it is possible that nicotinic activity in the ventral hippocampus could affect trace fear conditioning.

Scientific Question (Design):

These studies investigated the effects of ventral hippocampal nicotine infusion on trace and contextual fear conditioning. Initially, I generated a dose response curve to determine the optimal infusion dose of nicotine in the ventral hippocampus for producing effects on trace fear conditioning. Subsequently, I used the optimal dose (0.35 μg/side) to

determine if nicotine was diffusing medial to the ventral hippocampus to exert its effects. Additionally, to determine if nicotine‘s effects in the ventral hippocampus were particular to this training protocol, I infused nicotine into the ventral hippocampus prior to a 2-pairing trace fear conditioning procedure. Then, to determine if nicotine was also affecting cued learning, I infused nicotine into the ventral hippocampus prior to delay fear conditioning, with two different training protocols to rule out the possibility of over-training masking deficits. Finally, we infused nicotine prior to either training or testing of trace fear conditioning to determine if ventral hippocampal nicotine altered acquisition or expression of trace fear conditioning.

Methods

Subjects

These experiments used 149 8-12 week old male C57BL/6J mice. All mice were singly housed in standard colony cages, maintained on a 12h light/dark cycle with lights on at 7:00 am, and allowed *ad libitum* access to food and water. Housing, surgical, and behavioral procedures were approved by the Temple University Animal Care and Use Committee, and were in accordance with ethical standards of the APA.

Materials

Drugs and Infusion

Nicotine hydrogen tartrate salt [reported as freebase] 0.045, 0.09, 0.18, & 0.35 μg/side, obtained from Sigma-Aldrich (St. Louis, MO), was directly infused through 22-

gauge cannula. During infusions mice were gently restrained; then stainless steel stylets were removed from guide cannula and replaced with infusion cannula. Drugs were infused at a rate of 0.50 µl/min and at an injection volume of 0.50 µl per side. Infusion cannula were attached to polyethylene tubing (PE50; Plastics One) attached to a 10 µl Hamilton syringe (Reno, NV), which was controlled by a microinfusion pump (KDS 100; KD Scientific, New Hope, PA). Injection cannula were left in place for 30 seconds after infusion. Nicotine was infused immediately before training and/or testing. Spread of infusion using this procedure has been previously estimated to be ~1mm^3 (Lewis & Gould, 2007).

Apparatus

Training was conducted in conditioning chambers (model 307AW, Med Associates, St. Albans, VT) housed in sound attenuating cubicles. An 85 dB white noise conditioned stimulus (CS) was administered through speakers attached to the right wall of each chamber. A 2 second, 0.57 mA footshock unconditioned stimulus (US) was administered with a shock generator and scrambler (Med-Associates) through the chamber floors, which were composed of 18 stainless steel bars. 69 dB background noise was provided by 50 mm ventilation fans, mounted on the right wall of each sound-attenuating cubicle. Stimulus administration was controlled by an IBM-compatible PC running Med-PC software (Med-Associates).

Testing of trace fear conditioning was conducted in four conditioning chambers situated in sound attenuating cubicles located in a different room than that used for training. The testing chambers were distinct from the training chambers and had white

plastic floors, stainless steel sides, and Plexiglas panels for the front, rear and lid. Additionally, a novel olfactory cue (artificial vanilla extract) was applied to paper toweling placed below each of the chamber floors. Ventilation fans mounted on the right wall of the sound attenuating cubicles provided background noise. CS was generated with a Grason-Stradler noise generator (model 901B, West Concord, MA) attached to 3-inch speakers mounted on the left side of each of the conditioning chambers.

Procedure

Surgical

For surgical procedures mice were anesthetized with isoflurane, and placed in a mouse stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). The scalp was shaved, scrubbed ad retracted to expose the skull. Holes were drilled for anterior and lateral coordinates, determined from Paxinos and Franklin (2001) with respect to bregma, guide cannula were lowered into place with the stereotaxic, to match D/V coordinates, and permanently secured with dental cement. Ventral hippocampal coordinates were A/P –2.8, M/L 3.0, D/V 4.0; medial M/L 1.5. All cannula were purchased from Plastics One (Roanoke, Virginia). Stainless steel stylets were placed in the guide cannula to maintain patency during the 5-day recovery period. All animals received postoperative analgesic/anti-inflammatory ketprofen (2 mg/kg, sc; Fort Dodge, Fort Dodge, IA). 5 mice that showed post-surgical complications were excluded from further procedures.

Behavioral

Trace Fear Conditioning: Behavioral procedures were based on previous studies (Davis & Gould, 2007; Gould *et al.*, 2004; Raybuck & Gould, 2009). Training of trace fear conditioning was conducted during a single 16-minute training session wherein the mice were presented 5 CS-US pairings separated by a variable inter-trial-interval (90-120 seconds). CS-US parings consisted of a 30 second, 85 dB white noise CS presentation, followed by a 30 second trace interval, and terminated with the presentation of a 2 second, 0.57 mA footshock US. The training session began with activation of the house light and terminated 30 seconds following the last US presentation, at which point the house light was extinguished and mice were placed in their home cage. Twenty-four hours following training, mice were tested for both contextual and trace-cued associative conditioning. To test for contextual associations formed during the training session mice were placed in the training context and observed for freezing for five minutes. One to two hours later, to test for trace-cued conditioning, mice were placed in the altered testing chambers for six minutes, for the first three minutes no CS was presented and mice were scored for freezing to the altered context, a measure of generalized fear, then the CS was presented for three minutes and mice were scored for trace-cued freezing.

In addition to 5-pairing trace fear conditioning a subset of studies used a 2-pairing trace fear conditioning protocol, wherein mice received two CS-US pairings with a 30 second trace-interval and a 90 second inter-trial interval. These training sessions lasted 6 minutes and 30 seconds. All other parameters were as described above.

Delay Fear Conditioning: Delay fear conditioning procedures were based on previous studies (Davis & Gould, 2007; Gould et al., 2004). Training of delay fear conditioning was conducted during a single 5 minute and 30 second training session wherein the mice were presented with 2 CS-US pairings separated by a 90 second inter-trial-interval. CS-US parings consisted of a 30 second, 85 dB white noise CS presentation that co-terminated with a 2 second, 0.57 mA footshock US. The training session began with the activation of the house light and terminated 30 seconds following the last US presentation, at which point the house light was extinguished and mice were placed in their home cage. Testing of both contextual and delay-cued conditioning was performed as described for trace fear conditioning. In addition to 2-pairing delay conditioning, a subset of experiments used a 1-pairing delay fear conditioning paradigm, in which mice received only one CS-US pairing, and the CS was only 15 seconds in duration. Otherwise, training and testing parameters for both delay conditioning protocols were identical.

Histology

All brains were post-fixed in formalin for at least 24 hours, sliced on a cryostat, mounted, and nissl stained. Infusion coordinates were confirmed with either dye infusion, or by observing gliosis along the infusion cannula tracts.

Analysis

Analysis was conducted with SPSS 17. Data were analyzed with one-way ANOVA, followed with Tukey's HSD post hoc tests or with an independent-samples t-

test. Data sets not meeting homogeneity of variance assumption of the ANOVA were followed up with a Games-Howell post-hoc test.

Results

Placements

Histological analysis of all ventral hippocampal nicotine infusions showed that out of 149 surgical cannula implantations none were outside of the target area. Diagrams of placements for each study are displayed with the behavioral data.

Effects of ventral hippocampal nicotine infusion on trace fear conditioning

Multiple studies suggest that the ventral hippocampus is involved in trace fear conditioning and that the ventral hippocampus is a site for the modulation of learning by nicotine (Arthur & Levin, 2002; Bancroft & Levin, 2000; Bettany & Levin, 2001; Esclassan *et al.*, 2009; Kim & Levin, 1996; Levin *et al.*, 1999; Levin *et al.*, 2003; Pocivavsek *et al.*, 2006; Yoon & Otto, 2007). Thus, to determine if the ventral hippocampus was a likely site for the effects of nicotine on trace fear conditioning, I infused a range of doses of nicotine into this structure prior to training and testing of trace fear conditioning. ANOVA showed that infusion of nicotine into the ventral hippocampus affected contextual [$F(4, 32) = 5.758$, $p < 0.001$] and trace [$F(4,32) = 5.239$, $p < 0.002$] conditioning, but not baseline or altered freezing, Figure 8. Post hoc analysis revealed that ventral hippocampal nicotine infusion at doses of 0.18 and 0.35

µg/side produced deficits in contextual conditioning and that nicotine at 0.35 µg/side produced deficits in trace conditioning.

Figure 8.

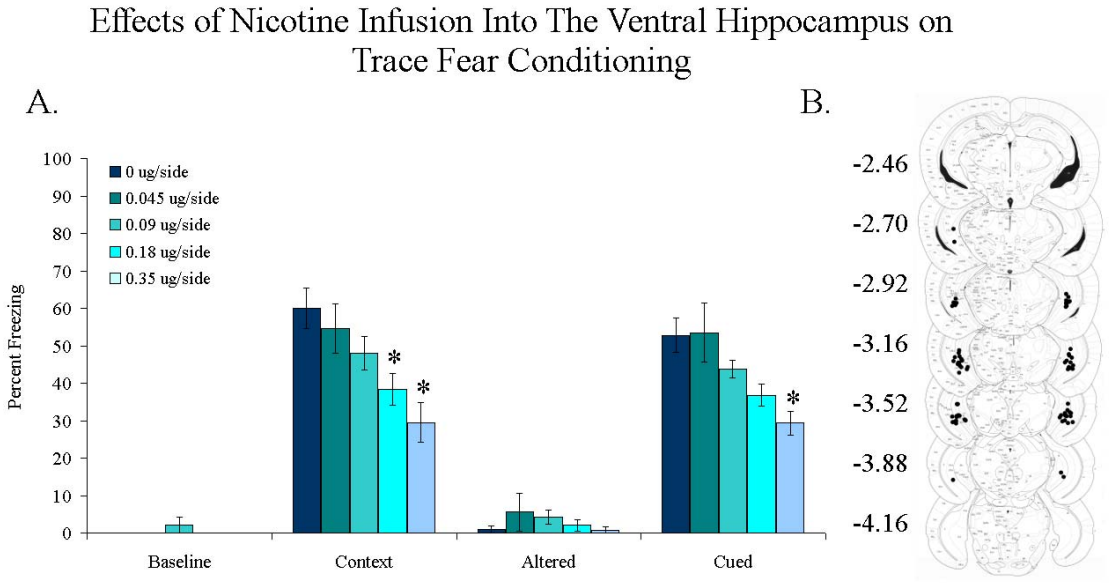

Figure 8: Nicotine infusion into the ventral hippocampus produced deficits in trace and contextual fear conditioning. A. **Infusion of nicotine into the ventral hippocampus produced dose-dependent deficits in trace (0.18 and 0.35 µg/side) and contextual (0.35 µg/side) conditioning. Significant difference ($p < 0.05$) from saline treated control group denoted with (*) data are reported as mean ± standard error of the mean. Subjects per groups were 6 (0 µg), 8 (0.045, 0.09, 0.18 µg), and 7 (0.35 µg).** B. **Histological analysis confirmed that all infusions were directed into the ventral hippocampus.**

Effects of nicotine infusion medial to the ventral hippocampus on trace fear conditioning

Infusion of nicotine into the ventral hippocampus produced deficits in trace and contextual fear conditioning. However, it is possible that nicotine's effects are due to diffusion into an adjacent brain structure. While a ventricle lies between the ventral hippocampus and adjacent cortical areas, and thus, it is unlikely that nicotine's effects in

the ventral hippocampus were due to diffusion into the perirhinal or entorhinal cortices, there is no barrier between the ventral hippocampus and medial structures such as the substantia nigra and the ventral tegmental area. To determine if nicotine produced deficits in trace fear conditioning by diffusing medially from the ventral hippocampus, nicotine at an effective dose (0.35 µg/side) was infused 1.5 mm medial to the ventral hippocampal coordinates prior to training and testing of trace fear conditioning. This placement was chosen because it places the infusion cannula approximately 1 mm from the ventral hippocampus. Infusion of nicotine (0.35 µg/side) medial to the ventral hippocampus had no effect, Figure 9.

Figure 9.

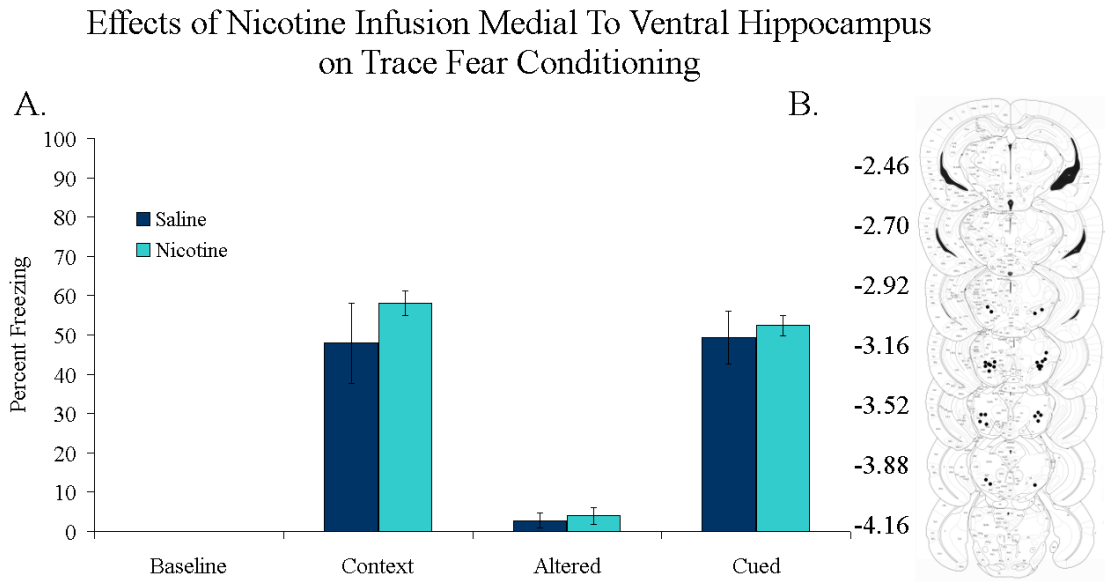

Figure 9: A. **Infusion of nicotine medial to the ventral hippocampus had no effect on trace or contextual fear conditioning. Thus, it is unlikely that effects of ventral hippocampal nicotine infusion on trace conditioning are due to infusion into brain regions medial to the ventral hippocampus, such as the substantia nigra and ventral tegmental area. There were 8 subjects per group.** B. **Histological analysis confirmed that all infusions were directed into the ventral hippocampus.**

Effects of ventral hippocampal nicotine infusion on 2-pairing trace fear conditioning

Infusion of nicotine into the ventral hippocampus produces deficits in trace and contextual fear conditioning. However, most studies of contextual fear conditioning train with 2 CS-US pairings, not with the 5 pairings typically used for trace conditioning. Thus, to determine if effects of ventral hippocampal nicotine infusion on trace conditioning are particular to this training protocol, and to facilitate comparison of these findings with those from other studies of contextual conditioning, I infused 0.09 and 0.35 μg/side nicotine into the ventral hippocampus prior to training and testing of 2-pairing trace fear conditioning. ANOVA showed that infusion of nicotine into the ventral hippocampus affected contextual [$F(2,22) = 18.950$, $p < 0.000$] and trace [$F(2,22) = 5.371$, $p < 0.013$] conditioning, but not baseline or altered freezing. Post hoc analysis revealed that infusion of 0.09 or 0.35 μg/side produced deficits in contextual conditioning, but only the 0.35 μg/side nicotine produced deficits in the trace conditioning, although 0.09 μg/side had a near significant effect ($p = 0.0811$).

Figure 10.

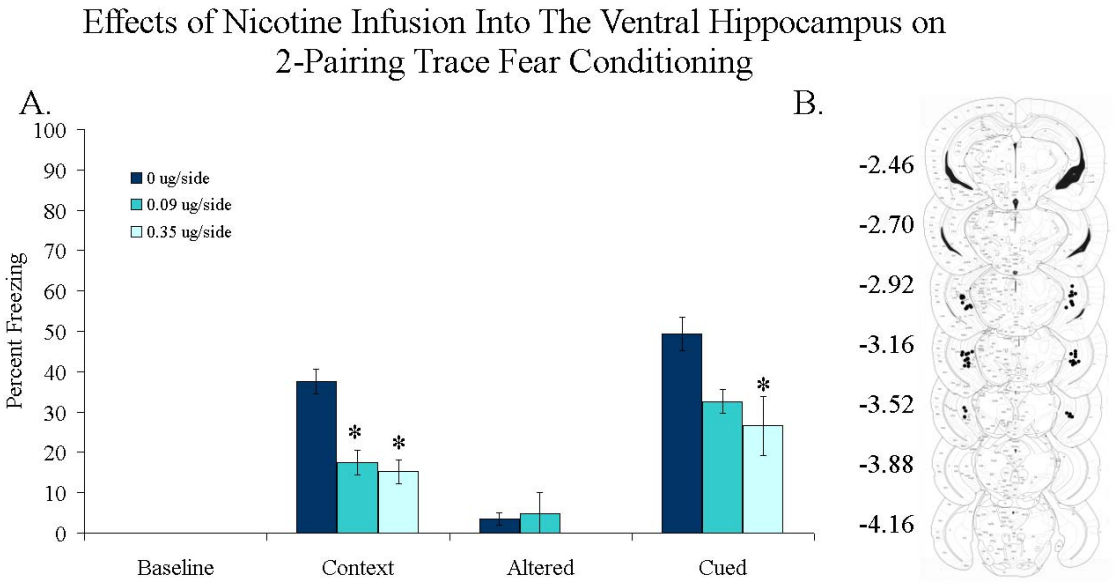

Figure 10: Ventral hippocampal nicotine infusion produces deficits in trace fear conditioning. A. **Infusion of nicotine into the ventral hippocampus produced deficits in contextual (0.09 & 0.35 µg/side) and trace (0.35 µg/side) conditioning in a 2-pairing trace fear conditioning paradigm. Significant difference ($p < 0.05$) from saline treated control group denoted with (*) data are reported as mean ± standard error of the mean. Subjects per group were 8 (0.0, 0.09 µg) and 9 (0.35 µg).** B. **Histological analysis confirmed that all infusions were directed into the ventral hippocampus.**

Effects of ventral hippocampal nicotine infusion on delay fear conditioning

2-pairing delay fear conditioning

The present findings demonstrate that infusion of nicotine into the ventral hippocampus produces deficits in trace and contextual fear conditioning, two tasks that critically depend on hippocampal integrity (McEchron *et al.,* 1998; Phillips & LeDoux, 1992). However, multiple studies suggest that the ventral hippocampus is involved in delay fear conditioning as well as trace and contextual fear conditioning (Anagnostaras *et al.,* 2001; Bast *et al.*, 2001; Burman *et al.*, 2006; Clark *et al.*, 1992; Esclassan *et al.*, 2009; Maren, 1999). Thus, to determine if deficits in trace fear conditioning produced by

nicotine infusion into the ventral hippocampus extend to delay fear conditioning (i.e. no trace interval), I infused nicotine at 0.09 and 0.35 µg/side into the ventral hippocampus prior to training and testing of delay fear conditioning. ANOVA showed that infusion of nicotine into the ventral hippocampus affected contextual [$F(3,27) = 10.768$, $p < 0.000$] conditioning, but not baseline or altered freezing, or delay conditioning. Post hoc analysis revealed that nicotine infusion at 0.09 or 0.35 µg/side produced deficits in contextual conditioning, Figure 11.

Figure 11.

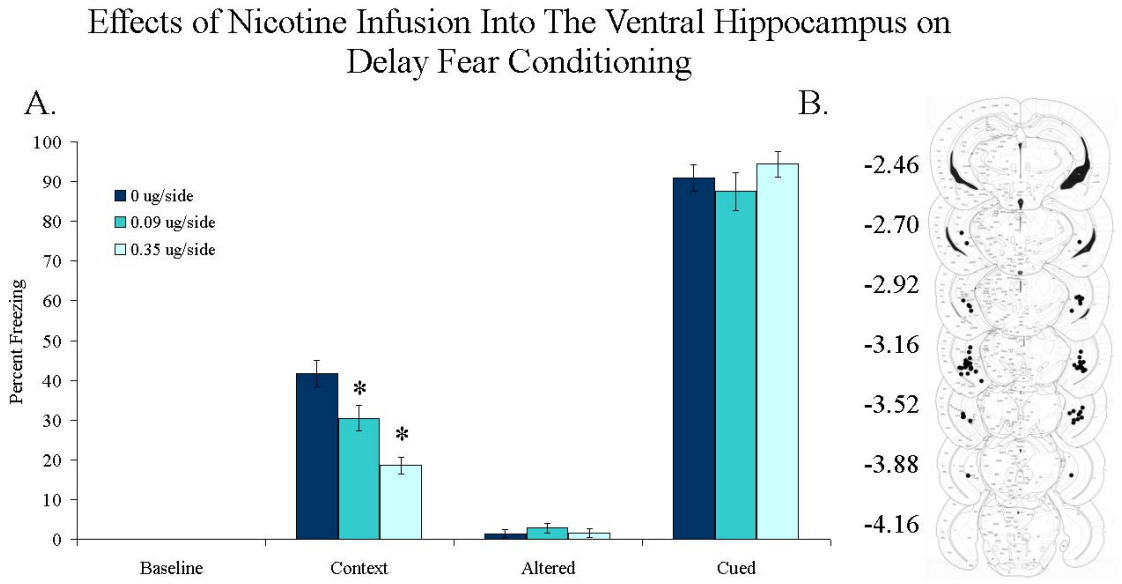

Figure 11. Ventral hippocampal nicotine infusion produces deficit in contextual but not delay fear conditioning. A. **Infusion of nicotine (0.09 or 0.35 µg/side) into the ventral hippocampus produced deficits in contextual fear conditioning, but had no effect on delay conditioning, using a 2-pairing 30s CS training protocol. Subjects per group were 8 (0.0, 0.09 µg) and 7 (0.35 µg).** B. **Histological analysis confirmed that all drug infusions were directed into the ventral hippocampus. Significant difference ($p < 0.05$) from saline treated control group denoted with (*) data are reported as mean ± standard error of the mean.**

1-pairing delay fear conditioning

Infusion of nicotine into the ventral hippocampus (0.35 μg/side) produces deficits in contextual conditioning but has no effect on delay fear conditioning. However, it is possible that effects of ventral hippocampal nicotine on delay fear conditioning could be masked by high freezing levels of mice conditioned with a 2-pairing, 30s CS delay conditioning protocol. Thus, I used a weaker conditioning protocol with 1 CS-US pairing and a 15s CS duration to evaluate the effects of 0.35 μg/side nicotine infusion into the ventral hippocampus, a dose that produces deficits in trace fear conditioning. This protocol has been shown to produce lower levels of freezing, preventing over training from obscuring possible drug effects (Gould *et al.*, 2004). To determine if the lack of deficits in delay conditioning was because over conditioning, I infused 0.35 μg/side nicotine prior to training and testing of 1-pairing delay fear conditioning. T-tests showed that infusion of 0.35 μg/side nicotine prior to 1-pairing delay conditioning into the ventral hippocampus produced deficits in contextual conditioning [$t(1,14) = 7.341$, $p < 0.000$] had no effect on baseline, altered, or delay freezing, Figure 12.

Figure 12.

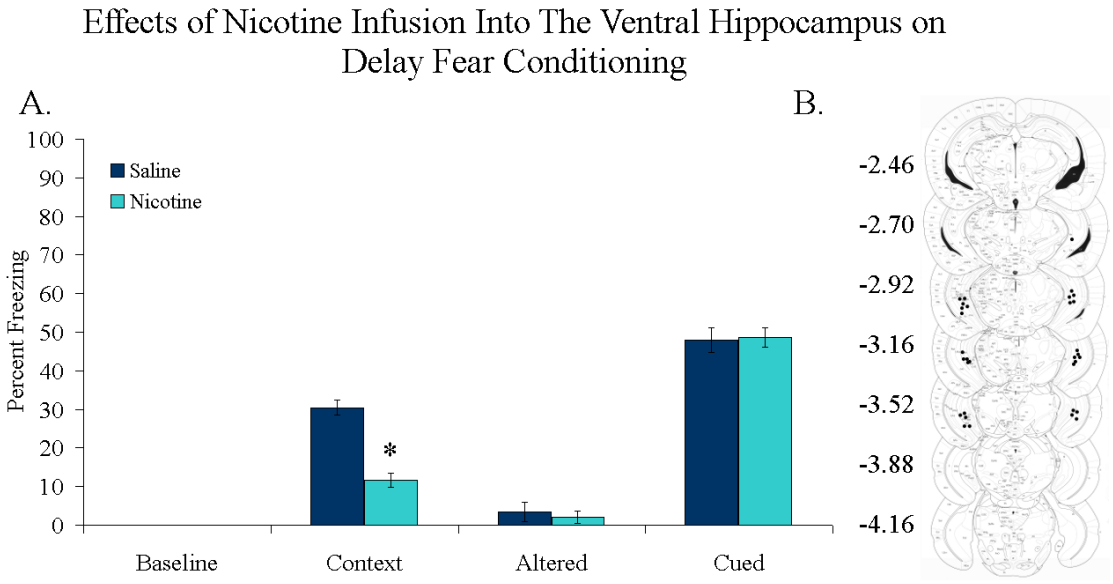

Figure 12: Ventral hippocampal nicotine infusion produces deficit in contextual but not delay fear conditioning. A. **Infusion of nicotine (0.35 µg/side) into the ventral hippocampus produced deficits in contextual fear conditioning, but had no effect on delay conditioning, using a 1-pairing 15s CS training protocol. Significant difference ($p < 0.05$) from saline treated control group denoted with (*) data are reported as mean ± standard error of the mean. There were 8 subjects per group.** B. **Histological analysis confirmed that all drug infusions were directed into the ventral hippocampus.**

Effects of nicotine in the ventral hippocampus at training or testing of trace fear conditioning

Infusion of nicotine in to the ventral hippocampus produced deficits in trace and contextual fear conditioning, regardless of training protocol. Effects of dorsal hippocampal nicotine infusion occur at training but not at testing. However, studies report that the ventral hippocampal involvement in trace conditioning involves expression of trace fear conditioning (Burman *et al.,* 2006; Esclassan *et al.*, 2009), although studies have reported effects of reversible inactivation of the ventral hippocampus at training (Rogers *et al.,* 2006; Yoon & Otto, 2007). Thus, to determine if ventral hippocampal

nicotine infusion affects processes supporting acquisition or expression of trace and contextual fear conditioning, I infused nicotine at 0.35 µg/side into the ventral hippocampus prior to either training or testing of 5-pairing trace fear conditioning (5 CS-US pairings). ANOVA showed that infusion of 0.35 µg/side nicotine into the ventral hippocampus at either training or testing affected contextual [$F(2,19) = 16.019$, $p < 0.000$] and trace [$F(2,19) = 6.281$, $p < 0.008$] conditioning, but not baseline or altered freezing, Figure 13. Post hoc analysis revealed that nicotine infusion at training, testing, or on both days produced deficits in both contextual and trace fear conditioning.

Figure 13.

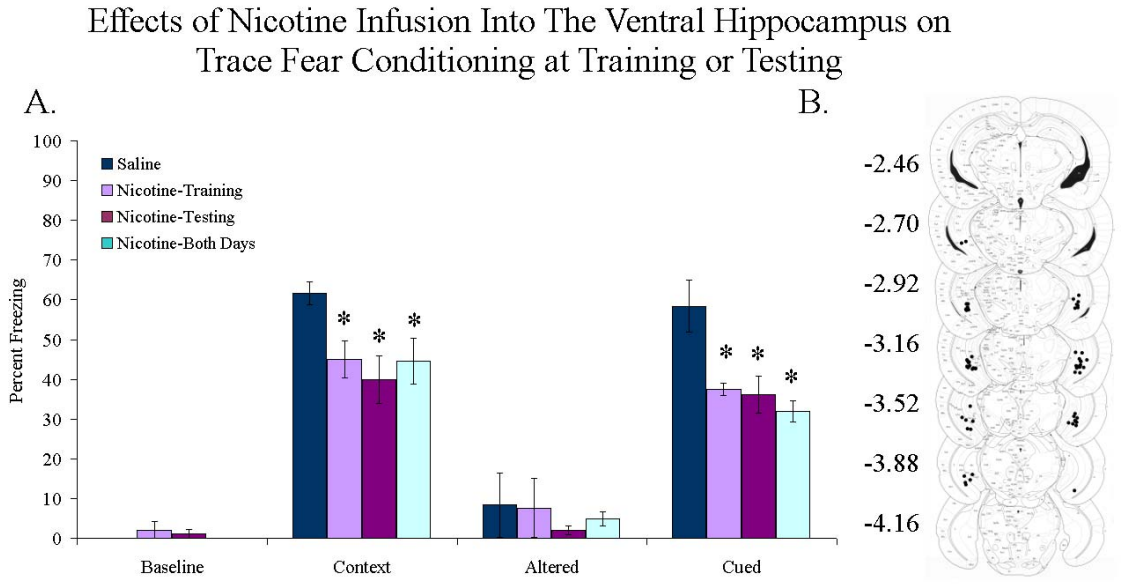

Figure 13: Ventral hippocampal nicotine infusion interferes with both acquisition and retrieval of trace and contextual fear conditioning. A. **Infusion of nicotine (0.35 µg/side) into the ventral hippocampus at training or testing, or at both training and testing of trace fear conditioning produced deficits in both trace and contextual fear conditioning. Thus, nicotinic stimulation of the ventral hippocampus interferes with both acquisition and retrieval of trace and contextual fear conditioning. There were 8 subjects per group.** B. **Histological analysis confirmed that all drug infusions were directed into the ventral hippocampus. Significant difference ($p < 0.05$) from saline treated control group denoted with (*) data are reported as mean ± standard error of the mean.**

Conclusions

These studies demonstrate that nicotine infusion into the ventral hippocampus produces deficits in acquisition and retrieval of trace and contextual fear conditioning. These findings are in stark contrast to the effects of nicotine in the dorsal hippocampus, where administration enhances both trace and contextual conditioning. Additionally, infusion of nicotine medial to the ventral hippocampus has no effect on conditioning, demonstrating that nicotine's effects are not due to drug diffusion into adjacent areas. Further, these effects occur across different training protocols, but do not extend to delay-cued conditioning, suggesting that they are not due to effects on processes such as anxiety or locomotion that would affect delay fear conditioning.

Ventral hippocampal nicotine infusion produces deficits in trace and contextual conditioning, but not in delay conditioning.

While lesions to the ventral hippocampus produce deficits in multiple forms of fear conditioning, the present findings show that nicotine infusion into this region produces deficits selective to trace and contextual fear conditioning. This finding may suggest a specialized function for nAChRs in the ventral hippocampus.

Lesions to the ventral hippocampus produce deficits in trace, contextual and delay fear conditioning (Czerniawski *et al.,* 2009; Esclassan *et al.*, 2009; Maren, 1999; Trivedi & Coover, 2004; Yoon & Otto, 2007). Additionally ventral hippocampal lesions have been shown to alter anxiety and response to an unconditioned stimulus (Bannerman *et al.,* 2003; Pentkowski *et al.*, 2006). These findings suggest that the ventral hippocampus is involved in expression of conditioned fear. In contrast to these reports, the present

findings show that nicotine infusion into the ventral hippocampus produces deficits selective to trace and contextual fear conditioning. These effects are consistent with a hypothesized role for the ventral hippocampus as a conduit for information between the dorsal hippocampus and amygdala (Maren & Fanselow, 1995). This role for the ventral hippocampus is supported by the fact that lesions to the ventral hippocampus reduce contextual fear conditioning associated synaptic plasticity in the dorsal hippocampus and amygdala (Maren & Fanselow, 1995; Maren & Holt, 2004), and by the fact that lesions to the ventral hippocampus produce deficits in retrieval of context specific extinction learning (Hobin *et al.*, 2006).

Since ventral hippocampal lesions produce deficits in trace fear conditioning as well as contextual fear conditioning, it may be that the ventral hippocampus serves as a conduit between the dorsal hippocampus and amygdala for temporal as well as contextual information. Thus, it may be that nicotine is suppressing information flow from the dorsal hippocampus to the amygdala. This action would prevent both acquisition and retrieval of trace and contextual fear conditioning. However, this hypothesis does not account for why ventral hippocampal lesion, or inactivation produces deficits in delay fear conditioning as well as trace and contextual conditioning. A number of studies have shown that different ventral hippocampal manipulations have different effects on delay fear conditioning. For instance, ventral hippocampal inactivation with tetrodotoxin produces deficits in contextual and cued components of delay conditioning, while inactivation with muscimol only affects contextual conditioning (Bast *et al.*, 2001). Although another study reports that muscimol also produces deficits in delay conditioning (Esclassan *et al.*, 2009), this difference could be attributed to differences in

training protocol, as Bast and colleagues (2001) trained with 10 CS-US pairings, whereas Esclassan and colleagues (2009) trained with 5 CS-US pairings. Additionally, NMDA administration blocked both contextual and cued conditioning, but the NMDA receptor antagonist MK-801 only blocked contextual conditioning (Zhang *et al.*, 2001). In this study, NMDA was administered at low concentration with the goal of activating NMDA receptors; however, it is notable that this drug is used at higher concentrations to produce excitotoxic lesions, thus it is possible that NMDA administration in this study could have produced lesions to the ventral hippocampus. These findings suggest that lesions to the ventral hippocampus produce deficits in delay fear conditioning, but other manipulations, NMDA receptor antagonism, and inactivation with muscimol may not affect delay conditioning. These effects may occur because the ventral hippocampus can modulate amygdala function, and lesions and inactivation result in different amount of disruption to the amygdala (Maren & Holt, 2004), whereas receptor antagonism may selectively affect information flow form the dorsal hippocampus, through the ventral hippocampus to the amygdala. It may be that nicotine infusion into the ventral hippocampus is disrupting information flow, without altering ventral hippocampal function enough to affect the amygdala and thus no change is seen in delay fear conditioning.

Effects of ventral hippocampal nicotine at both training and testing: A potential substrate of nicotine's systemic effects on contextual fear conditioning

The present findings show that nicotine infusion into the ventral hippocampus at either training or testing produces deficits in trace and contextual fear conditioning. These findings conflict with the effects of nicotine in the dorsal hippocampus, where

enhancement of trace and contextual conditioning occurs during training and they conflict with the effects of systemic nicotine on contextual conditioning, where enhancement occurs only if nicotine is administered at both training and testing (Gould & Higgins, 2003; Gould & Wehner, 1999). One possible reason for these discrepancies could be differences between infusion and systemic dosing. It is possible that the effective dose of nicotine in the ventral hippocampus following local infusion does not match the dose following systemic administration. However, since in preliminary studies deficits in trace and contextual fear conditioning were not seen at higher systemic doses, it seems unlikely that differences in dosing are sufficient to explain this discrepancy. Additionally, dose differences cannot explain differences between ventral hippocampal and dorsal hippocampal administration, where the same dose of nicotine in the ventral hippocampus produces deficits and in the dorsal hippocampus produces enhancement. Rather, the most likely explanation is that systemic nicotine affects multiple brain regions involved in these tasks and that enhancement of learning occurs because other brain regions out weigh contributions from the ventral hippocampus. Thus, effects of systemic nicotine on contextual fear conditioning may be explained as an interaction between nicotine's effects in the ventral hippocampus and nicotine's effects in the dorsal hippocampus.

Systemic and dorsal hippocampal nicotine both enhance trace and contextual fear conditioning (Davis & Gould, 2009; Davis *et al.*, 2007; Gould & Higgins, 2003; Gould & Wehner, 1999). However, enhancement by systemic administration depends on nicotine at both training and testing (Gould & Higgins, 2003; Gould & Wehner, 1999), while findings from Chapter 2 demonstrate that enhancement by dorsal hippocampal nicotine

occurs with administration only at training. Thus, it is likely that the necessity of nicotine at training and testing is due to nicotine's actions outside of the dorsal hippocampus. As deficits induced by nicotine infusion into the ventral hippocampus occur at either training or testing, this region may be involved in nicotine's systemic effects. However, enhancement of contextual fear conditioning by systemic nicotine does not depend on nicotine administration if tested one week post conditioning. This may be because intra-hippocampal consolidation has occurred such that retrieval no longer relies on nicotine's actions in the ventral hippocampus. Indeed, evidence from contextual fear conditioning suggests that there are phases of intra-hippocampal consolidation that could account for this. For instance retrieval of contextual fear conditioning one day post training depends on noradrenergic activity in the dorsal hippocampus, but four days after training retrieval is independent of dorsal hippocampal noradrenergic activity (Murchison *et al.*, 2004). Additionally, the dorsal and ventral hippocampus may be differentially involved in recent as opposed to remote memories, as retrieval of recent spatial learning activates both the ventral hippocampus and dorsal hippocampus, but retrieval of remote spatial learning only activates the dorsal hippocampus (Gusev *et al.*, 2005). Thus, necessity of systemic nicotine at both training and testing, but not one week following training may reflect intra-hippocampal consolidation that shifts retrieval away from ventral hippocampal dependence. However, further investigation will be necessary to determine if nAChR signaling in the ventral hippocampus plays a critical role in the effects of systemic nicotine on fear conditioning.

Summary

Nicotine infusion into the ventral hippocampus produces deficits in trace and contextual fear conditioning. These effects occur at either training or testing, but do not extend to delay conditioning, suggesting that they are not mediated through anxiety or locomotion, but rather that ventral hippocampal nAChRs can modulate information processing in trace and contextual fear conditioning that is critical during both acquisition and retrieval.

CHAPTER 4. THE EFFECTS OF MEDIAL PREFRONTAL CORTICAL NICOTINE ON TRACE FEAR CONDITIONING

Introduction

Dorsal and ventral hippocampal involvement on trace fear conditioning is well established, and Chapters 2 and 3 demonstrate that within these areas nicotine has effects on trace fear conditioning. However, multiple studies implicate the medial prefrontal cortex in trace fear conditioning, and studies also suggest that nicotine has effects in this brain area.

Multiple studies suggest that the medial prefrontal cortex is critical to trace fear conditioning. Neural activity in the medial prefrontal cortex is associated with trace fear conditioning. For instance, Zif 268 and c-Fos expression, markers of neuronal activity, increase in the medial prefrontal cortex following trace fear conditioning (Weitemier & Ryabinin, 2004). Additionally, in rats prefrontal cortical areas show sustained neuronal activity during the trace interval (Gilmartin & McEchron, 2005). Further, brain regions with functional and anatomical similarities to the rodent medial prefrontal cortex, such as the frontal operculum and middle frontal gyri, in humans show increased BOLD signal during trace fear conditioning, but not during delay conditioning (Knight *et al.*, 2004). Additionally, medial prefrontal cortical function is critical to trace fear conditioning. For instance, lesions to or temporary inactivation of the medial prefrontal cortex produce deficits in trace fear conditioning (Blum *et al.,* 2006; Quinn *et al.*, 2008). In addition, D1 dopaminergic receptor antagonist SCH-23390 produces deficits if infused into the medial

prefrontal cortex during trace fear conditioning (Runyan & Dash, 2004). ERK is activated in the medial prefrontal cortex following trace fear conditioning (Runyan & Dash, 2004), and ERK inhibitors produce deficits in acquisition of the trace conditioned CS-US association (Runyan *et al.*, 2004). Additionally, inhibition of protein transcription within the medial prefrontal cortex produces deficits in long-term memory of trace fear conditioning (Blum *et al.*, 2006). Furthermore, a recent study demonstrates that the medial prefrontal cortex is the final site of long-term memory storage for trace fear associations, as lesions to the medial prefrontal cortex at 200 days after training produced deficits in trace fear, while lesions to the hippocampus at this time point had no effect (Quinn *et al.*, 2008). Thus, the medial prefrontal cortex is critical to both acquisition and consolidation of trace fear conditioning.

Nicotine's effects in the medial prefrontal cortex suggest that it could be a site of action for nicotine's effects on trace fear conditioning. Nicotine enhances c-Fos production in the medial prefrontal cortex (Mathieu-Kia *et al.,* 1998; Nisell *et al.*, 1997; Pagliusi *et al.*, 1996; Pich *et al.*, 1997; Schilström *et al.*, 2000). Additionally, Nicotine enhances dopamine release in the media prefrontal cortex (Livingstone *et al.,* 2009; Shearman *et al.*, 2005; Zhu *et al.*, 2007; Zhu *et al.*, 2009)., an event critical to trace fear conditioning (Runyan & Dash, 2004), which is also thought to support working memory (Arnsten, 1997; Grace *et al.*, 2007; Romanides *et al.*, 1999). Further, decreases in dopaminergic activity in the VTA produced by inactivation of the medial prefrontal cortex are be ameliorated by nicotine (Svensson *et al.*, 1990). These effects may be behaviorally relevant since systemic nicotine administration increases low but not high rates of intra-cranial self stimulation in the medial prefrontal cortex (Arregui-Aguirre *et*

al., 1987; Vives & Mora, 1986). Additionally, the medial prefrontal cortex may be a critical mediator of nicotine's cognitive effects, as medial prefrontal nicotine infusion can substitute for systemic nicotine in drug discrimination studies (Miyata *et al.,* 1999; Miyata *et al.*, 2002; Smith & Stolerman, 2009). These effects of systemic and local nicotine administration on medial prefrontal cortical function, combined with the role of the medial prefrontal cortex in trace fear conditioning suggest that this area may be involved in nicotine's effects on trace fear conditioning.

Scientific Question (Design)

These studies investigated the effects of medial prefrontal cortical nicotine infusion on trace fear conditioning. Initially, I generated a dose response curve to determine the optimal infusion dose of nicotine in the medial prefrontal cortex for producing effects on trace fear conditioning. Subsequently, I used the optimal dose (0.09 μg/side) to determine if nicotine was diffusing above or below the medial prefrontal cortex to exert its effects. Additionally, to determine if nicotine's effects in the medial prefrontal cortex were particular to a 5-CS-US trace conditioning protocol, I infused nicotine into the medial prefrontal cortex prior to a 2-pairing trace fear conditioning procedure. Then, to determine if nicotine in the medial prefrontal cortex affected cued learning, I infused nicotine into the medial prefrontal cortex prior to delay fear conditioning, with two different training protocols. Finally, I infused nicotine prior to either training or testing of trace fear conditioning to determine if nicotine in the medial prefrontal cortex was enhancing processes related to acquisition or processes related to expression of trace fear conditioning.

Methods

Subjects

These experiments used 192 8-12 week old male C57BL/6J mice. All mice were singly housed in standard colony cages, maintained on a 12h light/dark cycle with lights on at 7:00 am, and allowed *ad libitum* access to food and water. Housing, surgical, and behavioral procedures were approved by the Temple University Animal Care and Use Committee, and were in accordance with ethical standards of the APA.

Materials

Drugs and Infusion

Nicotine hydrogen tartrate salt [reported as freebase] 0.045, 0.09, 0.18, & 0.35 μg/side was obtained from Sigma-Aldrich (St. Louis, MO). Drugs were directly infused through 33-gauge cannula. During infusions mice were gently restrained while stainless steel stylets were removed from guide cannula and replaced with infusion cannula. Drugs were infused at a rate of 0.50 μl/min and at an injection volume of 0.50 μl per side. Infusion cannula were attached to polyethylene tubing (PE50; Plastics One) attached to a 10 μl Hamilton syringe (Reno, NV), which was controlled by a microinfusion pump (KDS 100; KD Scientific, New Hope, PA). Injection cannula were left in place for 30 seconds after infusion. Nicotine was infused immediately before training and/or testing. Spread of infusion using this procedure has been previously estimated to be ~1mm^3 (Lewis & Gould, 2007).

Apparatus

Training was conducted in conditioning chambers (model 307AW, Med Associates, St. Albans, VT) housed in sound attenuating cubicles. An 85 dB white noise conditioned stimulus (CS) was administered through speakers attached to the right wall of each chamber. 69 dB background noise was provided by 50 mm ventilation fans, mounted on the right wall of each sound-attenuating cubicle. A 2 second, 0.57 mA footshock unconditioned stimulus (US) was administered through the chamber floors, which were composed of 18 stainless steel bars, with a shock generator and scrambler (Med-Associates). Stimulus administration was controlled by an IBM-compatible PC running Med-PC software (Med-Associates).

Testing of trace fear conditioning was conducted in four conditioning chambers situated in sound attenuating cubicles located in a different room than that used for training. The testing chambers were distinct from the training chambers and had white plastic floors, stainless steel sides, and Plexiglas panels for the front, rear and lid. Additionally, a novel olfactory cue (artificial vanilla extract) was applied to paper toweling placed below each of the chamber floors. Ventilation fans mounted on the right wall of the sound attenuating cubicles provided background noise. For testing, the CS was generated with a Grason-Stradler noise generator (model 901B, West Concord, MA) attached to 3-inch speakers mounted on the left side of each of the conditioning chambers.

Procedure

Surgical

For surgical procedures mice were anesthetized with isoflurane, and placed in a mouse stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). The scalp was shaved, scrubbed and retracted to expose the skull. Holes were drilled for anterior and lateral coordinates, determined from Paxinos and Franklin (2001) with respect to bregma, guide cannula were lowered into place with the stereotaxic, to match D/V coordinates, and permanently secured with dental cement. Medial prefrontal cortical coordinates were A/P 1.7, M/L 1.0, D/V 2.5, above D/V -1, below D/V –3.5, relative to bregma. All cannula were purchased from Plastics One (Roanoke, Virginia). Stainless steel stylets were placed in the guide cannula to maintain patency during the 5-day recover period. All animals received postoperative analgesic/anti-inflammatory ketprofen (2 mg/kg, sc; Fort Dodge, Fort Dodge, IA).

Behavioral

Trace Fear Conditioning: Behavioral procedures were based on previous studies (Raybuck & Gould, 2009) Davis & Gould, 2007; Gould et al., 2004). Training of trace fear conditioning was conducted during a single 16-minute training session wherein the mice were presented 5 CS-US pairings separated by a variable inter-trial-interval (90-120 seconds). CS-US parings consisted of a 30 second 85 dB white noise CS presentation, followed by a 30 second trace interval, and terminated with the presentation of a 2 second 0.57 mA footshock US. The training session began with activation of the house light and terminated 30 seconds following the last US presentation, at which point the house light

was extinguished and mice were placed in their home cage. Twenty-four hours following training, mice were tested for both contextual and trace-cued associative conditioning. To test for contextual associations formed during the training session mice were placed in the training context and observed for freezing for five minutes. One to two hours later, mice were placed in the altered testing chambers for six minutes, for the first three minutes no CS was presented and mice were scored for freezing to the altered context, a measure of generalized fear, then the CS was presented for three minutes and mice were scored for trace-cued freezing.

In addition to 5-pairing trace fear conditioning a subset of studies used a 2-pairing trace fear conditioning protocol, wherein mice received two CS-US pairings with a 30 second trace-interval and a 90 second inter-trial interval. These training session lasted 6 minutes and 30 seconds. All other parameters were as described previously.

Delay Fear Conditioning: Delay fear conditioning procedures were based on previous studies (Davis & Gould, 2007; Gould et al., 2004). Training of delay fear conditioning was conducted during a single 5 minute and 30 second training session wherein the mice were presented with 2 CS-US pairings separated by a 90 second inter-trial-interval. CS-US parings consisted of a 30 second 85 dB white noise CS presentation that co-terminated with a 2 second 0.57 mA footshock US. The training session began with the activation of the house light and terminated 30 seconds following the last US presentation, at which point the house light was extinguished and mice were placed in their home cage. Testing of both contextual and delay-cued conditioning was performed as described for trace conditioning. In addition to the 2-pairing delay conditioning

training protocols, a subset of experiments used a 1-pairing delay fear conditioning paradigm, in which mice received only one CS-US pairing, and the CS was only 15 seconds in duration. Otherwise, training and testing parameters for both delay conditioning protocols were identical.

Histology

All brains were post-fixed in formalin for at least 24 hours, sliced on a cryostat, mounted, and nissl stained. Infusion coordinates were confirmed with either dye infusion, or by observing gliosis along the infusion cannula tracts.

Analysis

Analysis was conducted with SPSS 17. Data were analyzed with one-way ANOVA followed with Tukey's HSD post hoc tests or with an independent-samples t-test. Data sets not meeting homogeneity of variance assumption of the ANOVA were followed up with a Games-Howell post-hoc test.

Results

Placements

Histological analysis of all medial prefrontal cortical infusions showed that out of 192 surgical cannula implantations none were outside of the target area. Diagrams of placements for each study are displayed with the behavioral data.

Effects of medial prefrontal cortical nicotine infusion on trace fear conditioning

Systemic nicotine administration enhances trace fear conditioning and nicotine infusion into the dorsal hippocampus enhances trace and contextual fear conditioning. However, the medial prefrontal cortex has also been implicated in trace fear conditioning. Thus, I infused nicotine into the medial prefrontal cortex prior to training and testing of trace fear conditioning across a range of doses to determine if nicotine's action in the medial prefrontal cortex may also support the effects of systemic nicotine on trace fear conditioning. ANOVA showed that infusion of nicotine into the medial prefrontal cortex significantly affected trace conditioning [$F(4,61) = 6.218$, $p < 0.000$], but did not affect contextual conditioning, baseline or altered freezing, Figure 14. Post hoc analysis revealed that mice treated with 0.09 μg/side or with 0.18 μg/side showed enhanced trace conditioning compared to controls.

Figure 14.

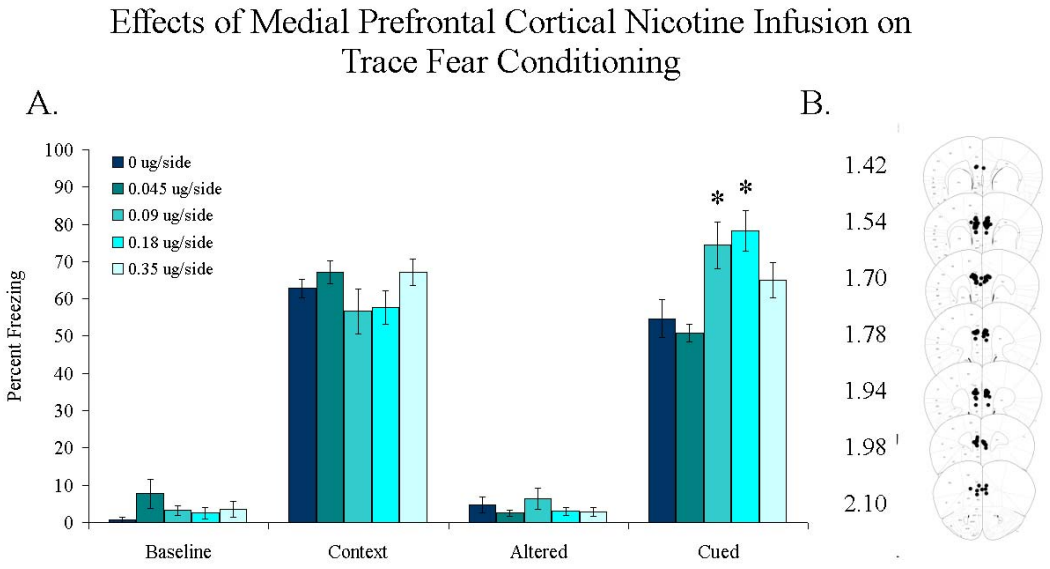

Figure 14: Medial prefrontal cortical nicotine infusion enhances trace fear conditioning. A. **Infusion of nicotine (0.09 & 0.18 µg/side) into the medial prefrontal cortex produces enhancement of trace fear conditioning, but has no effect on contextual fear conditioning. Thus, nicotine's effects on the medial prefrontal cortex are specific to trace conditioning. Significant difference ($p < 0.05$) from saline treated control group denoted with (*) data are reported as mean ± standard error of the mean. Subjects per group were 13 (0.0, 0.045, 0.09, 0.18 µg) and 14 (0.35 µg).** B. **Histological analysis confirmed that all infusions were directed into the medial prefrontal cortex**

Diffusion controls for the effects of medial prefrontal cortical nicotine on trace fear conditioning.

Above

Medial prefrontal cortical nicotine infusion selectively enhanced trace fear conditioning, however this effect could be due to nicotine diffusing up the cannula tract into areas immediately above the medial prefrontal cortex. Thus, to determine if nicotine's effects on trace fear conditioning were due to drug diffusion above the medial prefrontal cortex, I infused nicotine (0.09 µg/side) above the medial prefrontal cortex

prior to training and testing of trace fear conditioning. T-test showed that infusion of 0.09 µg/side nicotine had no effect on contextual or trace conditioning, or on baseline or altered freezing, Figure 15.

Figure 15.

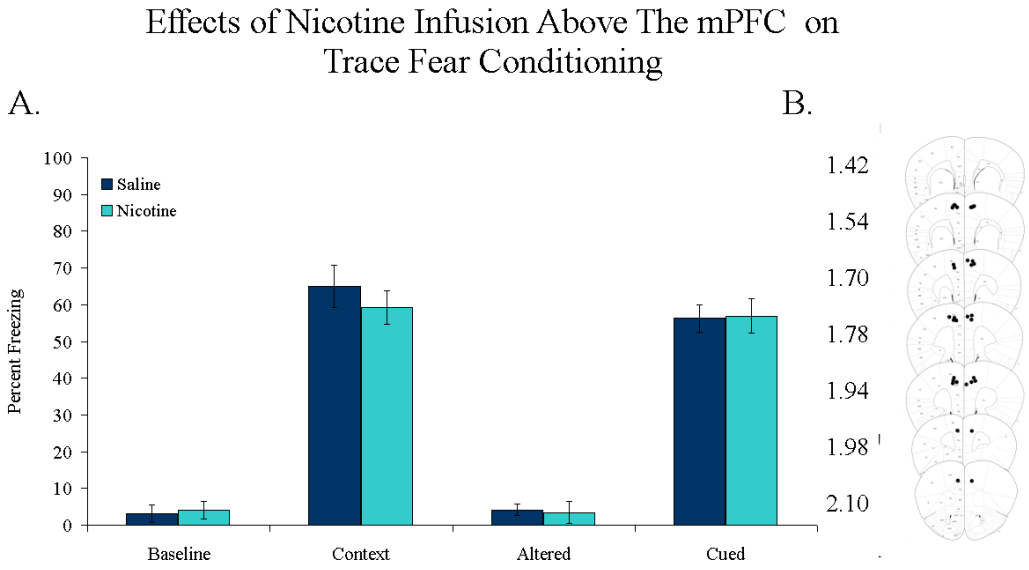

Figure 15: Nicotine infusion above the medial prefrontal cortex does not affect trace fear conditioning. A. **Infusion of nicotine (0.09 µg/side) directly above the medial prefrontal cortex had no effect on any measures of trace conditioning. There were 8 subjects per group.** B. **Histological analysis confirmed that all placements were in cortical areas directly above the medial prefrontal cortex.**

Below

Medial prefrontal cortical nicotine infusion selectively enhanced trace fear conditioning and infusion of nicotine above the medial prefrontal cortex had no effect on conditioning. However, it is possible that enhancement is due to nicotine diffusing to areas below the medial prefrontal cortex. Thus, to determine if nicotine's effects on trace

fear conditioning are due to drug diffusion into these areas, I infused nicotine (0.09 µg/side) below the medial prefrontal cortex prior to training and testing of trace fear conditioning. T-test showed that infusion of 0.09 µg/side nicotine had no effect on contextual or trace conditioning, or on baseline or altered freezing, Figure 16.

Figure 16.

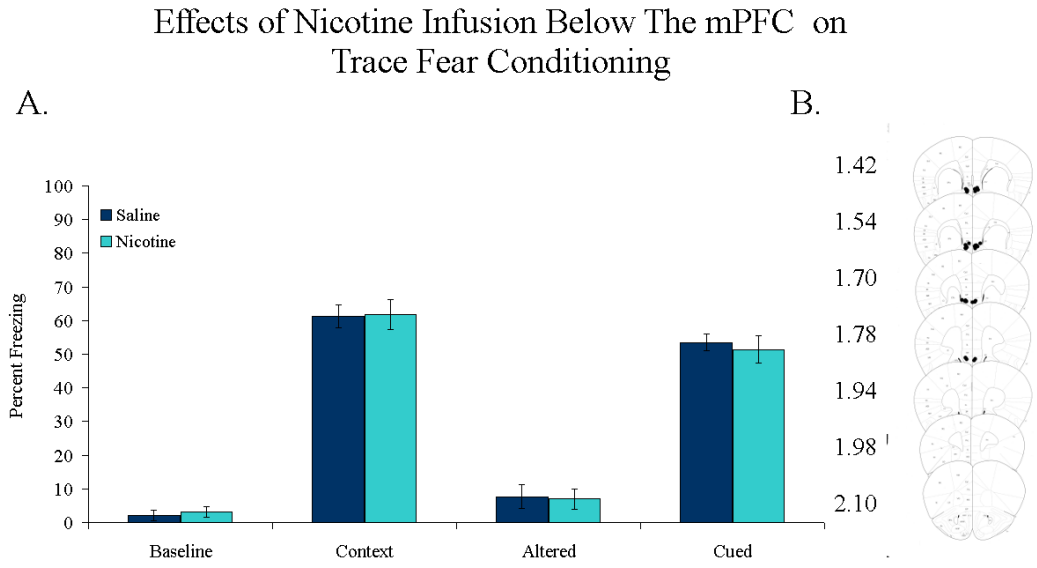

Figure 16. Infusion of nicotine below the medial prefrontal cortex had no effect on trace fear conditioning. A. **Infusion of nicotine (0.09 µg/side) directly below the medial prefrontal cortex had no effect on any measures of trace conditioning. There were 8 subjects per group.** B. **Histological analysis confirmed that all placements were in areas below the medial prefrontal cortex.**

Effects of medial prefrontal cortical nicotine on 2-pairing trace fear conditioning

Medial prefrontal cortical nicotine infusion enhances trace fear conditioning, but does not affect contextual fear conditioning. However, most studies of contextual fear conditioning train with 2 CS-US pairings, not with the 5 pairings used for trace conditioning. Additionally, as reported in chapter 2 effects of dorsal hippocampal

nicotine on contextual conditioning occur at higher doses with shifts in training protocol. Similar shifts in effective dose occurring in the medial prefrontal cortex could affect trace conditioning, or alter the effects of nicotine on contextual conditioning. Thus, to determine if effects of nicotine in the medial prefrontal cortex are particular to this training protocol, and to facilitate comparison of these findings with those from other studies of contextual conditioning, I used infused nicotine into the medial prefrontal cortex prior to training and testing of a 2-pairing trace fear conditioning protocol. ANOVA showed that nicotine infusion affected trace [$F(2,20) = 12.119, p < 0.000$] fear conditioning in this task, without affecting contextual conditioning or baseline or altered freezing. Post hoc analysis revealed that 0.09 μg/side nicotine enhanced 2-pairing trace fear conditioning, and that 0.35 μg/side had no effect, Figure 17.

Figure 17.

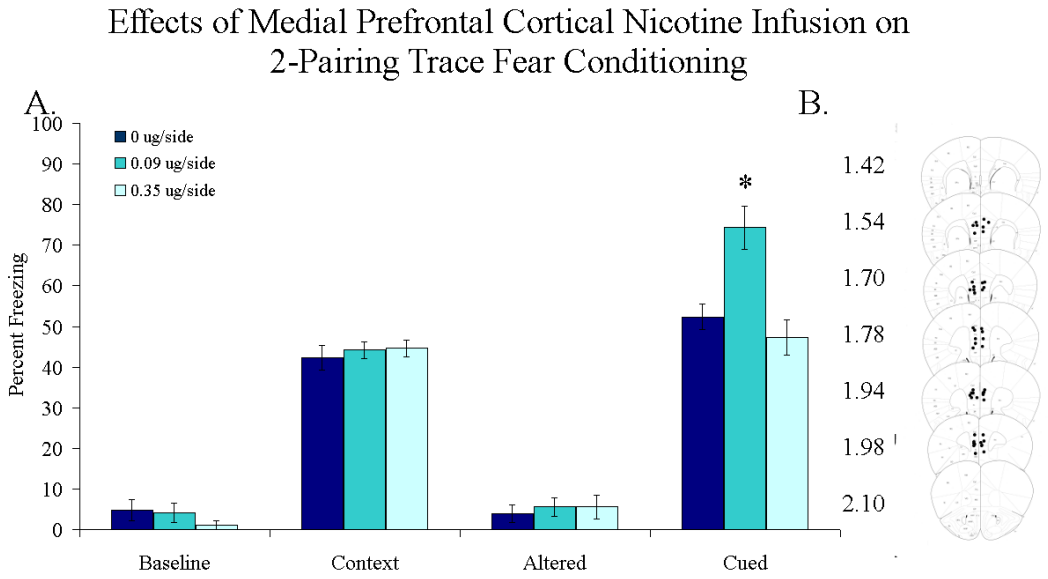

Figure 17: Medial prefrontal cortical nicotine infusion enhances trace fear conditioning, regardless of training protocol. A. **Infusion of nicotine (0.09 µg/side) into the medial prefrontal cortex enhanced trace fear conditioning, but had no effect on contextual fear conditioning in 2-pairing trace fear conditioning. Significant difference ($p < 0.05$) from saline treated control group denoted with (*) data are reported as mean ± standard error of the mean. Subjects per group were 7 (0.0 µg) and 8 (0.09 & 0.35 µg).** B. **Histological analysis confirmed that all infusions were directed into the medial prefrontal cortex.**

Effects of medial prefrontal cortical nicotine infusion on delay fear conditioning

2-pairing delay fear conditioning

Medial prefrontal cortical nicotine infusion enhances trace but not contextual fear conditioning, however it is possible that this effect is due to enhancement of stimulus salience by nicotine's action in the medial prefrontal cortex. If this were the case then medial prefrontal cortex nicotine infusion would be expected to affect delay fear conditioning as well as trace fear conditioning. Thus, to determine if medial prefrontal cortex nicotine infusion affects delay fear conditioning, I infused nicotine into the medial prefrontal cortex prior to training and testing of delay fear conditioning (i.e. no trace

interval). ANOVA showed no effect of nicotine infusion at 0.09 or 0.35 µg/side on contextual or delay conditioning, or baseline or altered freezing in this paradigm, Figure 18.

Figure 18.

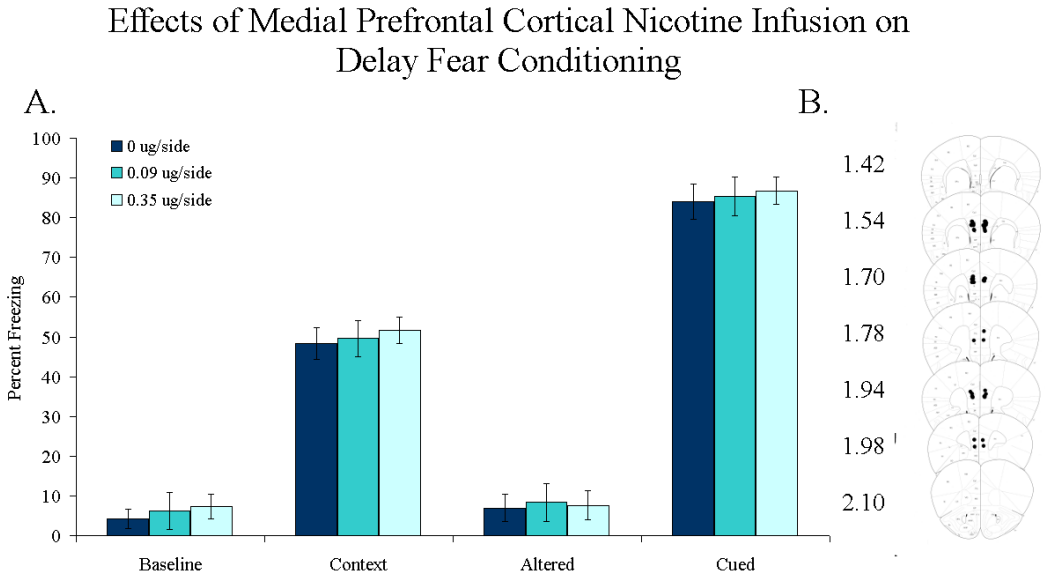

Figure 18: Medial prefrontal cortical nicotine infusion had no effect on delay fear conditioning. A. **Infusion of nicotine (0.09 or 0.35 µg/side) had no effect on contextual or delay fear conditioning, trained with 2 CS-US pairings, CS 30s. There were 8 subjects per group.** B. **Histological analysis confirmed that all infusions were directed into the medial prefrontal cortex.**

1-pairing delay fear conditioning

Nicotine infusion into the medial prefrontal cortex had no effects on delay fear conditioning, but it is possible that effects may have been obscured by the high levels of delay freezing in these mice. Thus, to determine if nicotine infusion in to the medial prefrontal cortex has effects on delay fear conditioning, I also infused nicotine (0.09 µg/side) prior to training and testing of a 1 CS-US pairing, 15s CS delay conditioning protocol. T-test showed that infusion of nicotine into medial prefrontal cortex had no

effect on delay or contextual conditioning, or baseline or altered freezing following training with a 1 CS-US presentation, 30s CS protocol, Figure 19.

Figure 19.

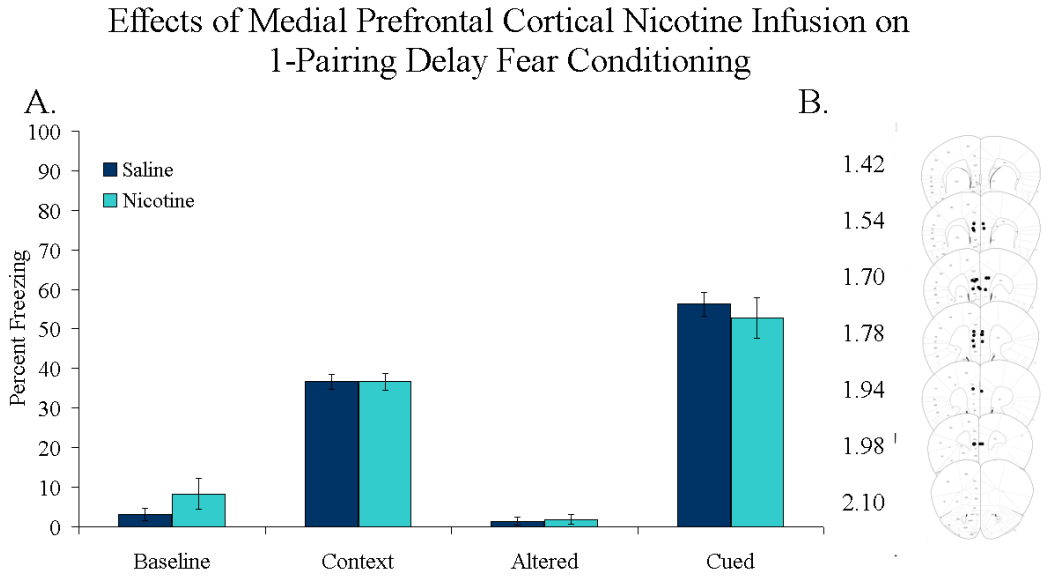

Figure 19: Medial prefrontal cortical nicotine infusion has no effect on delay fear conditioning. A. **Infusion of nicotine (0.09 µg/side) had no effect on contextual or delay fear conditioning, trained with 1 CS-US pairings, CS 15s. Subjects per group were 8 (Saline) and 7 (Nicotine).** B. **Histological analysis confirmed that all infusions were directed into the medial prefrontal cortex.**

Effects of medial prefrontal cortical nicotine infusion at training or testing of trace fear conditioning

Nicotine infusion into the medial prefrontal cortex selectively enhances trace fear conditioning, but the medial prefrontal cortex is critically involved in both acquisition and retrieval of trace fear conditioning (Quinn *et al.*, 2008). Thus, it is not clear whether enhancement by nicotine is due to the actions of nicotine on processes underlying acquisition or processes underlying retrieval of trace conditioning. To determine if medial prefrontal cortical nicotine infusion enhances acquisition or expression of trace

conditioning (5 CS-US pairings), I infused nicotine (0.09 µg/side) into the medial prefrontal cortex prior to training, testing, or both training and testing of trace fear conditioning. ANOVA showed that infusion of nicotine at training, testing, or both training and testing at a dose of 0.09 µg/side affected trace conditioning [$F(3,28) = 6.530$, $p < 0.002$], but had no effect on contextual conditioning or baseline or altered freezing, Figure 20. Post hoc analysis revealed that administration of nicotine at 0.09 µg/side enhanced trace conditioning if administered prior to training, or prior to both training and testing, but not prior to testing alone.

Figure 20.

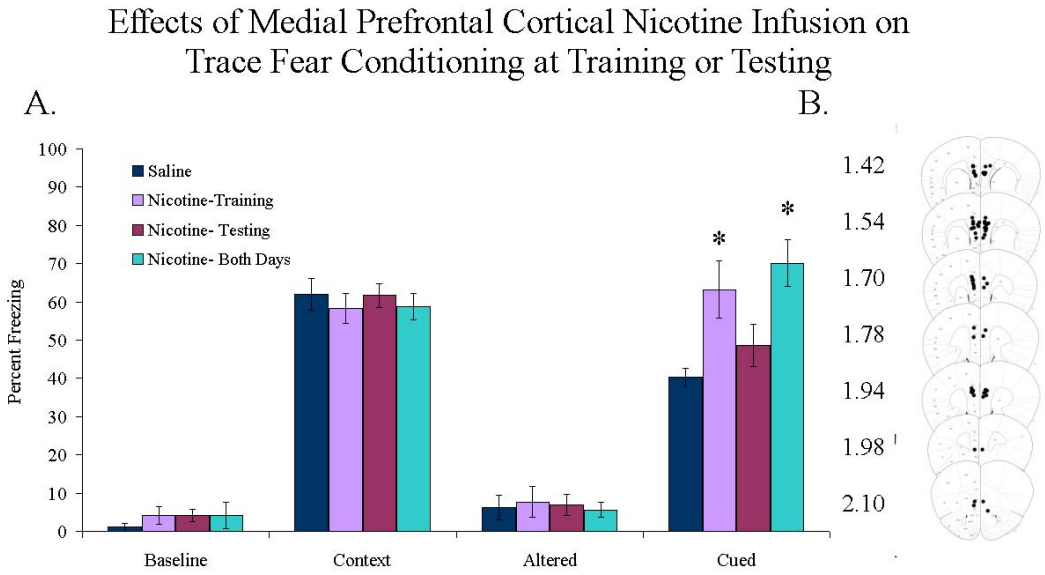

Figure 20: Enhancement of trace fear conditioning by medial prefrontal cortical nicotine infusion occurs at training but not at testing. A. **Infusion of 0.09 µg/side nicotine into the medial prefrontal cortex at training or at training and testing of trace fear conditioning was sufficient to enhance trace conditioning. However, infusion at testing had no effect. Thus, nicotine's effects on trace fear conditioning in the medial prefrontal cortex occur at training, but not at testing. Significant difference ($p < 0.05$) from saline treated control group denoted with (*) data are reported as mean ± standard error of the mean. There were 8 subjects per group.** B. **Histological analysis confirmed that all infusions were directed into the medial prefrontal cortex.**

Conclusions

The present findings demonstrate that infusion of nicotine in to the medial prefrontal cortex enhances acquisition of trace fear conditioning, but does not affect contextual or delay conditioning.

Medial prefrontal cortical nicotine infusion enhances trace fear conditioning

Enhancement of trace conditioning by medial prefrontal cortical nicotine infusion occurs at doses similar to those that enhance trace conditioning in the dorsal hippocampus. This may suggest that effects of nicotine in the dorsal hippocampus and medial prefrontal cortex occur through similar mechanisms, either that they occur through the same class of nicotinic receptors, or that they occur through a common secondary mechanism, such as dopamine release or ERK phosphorylation. Both medial prefrontal cortex and dorsal hippocampus are critically involved in trace fear conditioning, as lesions to either structure pre- or post-training produce deficits in trace conditioning (Blum *et al.,* 2006; Quinn *et al.*, 2008). Additionally, ERK phosphorylation occurs in both structures following trace fear conditioning, and inhibition of ERK activation in either structure produces retention deficits, but does not affect behavior during acquisition (Runyan *et al.*, 2004), suggesting that this event is critical for consolidation of learning. Additionally, both structures show increases in c-Fos and Zif 268 production following trace fear conditioning (Weitemier & Ryabinin, 2004). Thus, it may be that similar events in each brain structure support trace fear conditioning. Further, nicotine has similar effects in both of these structures. For instance, nicotine administration increases dopamine efflux in both medial prefrontal cortex and dorsal

hippocampus (Livingstone *et al.,* 2009; Shearman *et al.*, 2005; Singer *et al.*, 2004), and nicotine increases ERK phosphorylation in these structures as well (Valjent *et al.*, 2004). Indeed, there may be a relationship between these events, dopamine release and ERK activation, as antagonism of dopaminergic D1 receptors reduces nicotine induced ERK activation in these areas, and D1 receptor antagonism in the medial prefrontal cortex produces deficits in trace fear conditioning (Runyan & Dash, 2004), although the role of D1 receptors in the dorsal hippocampus has not yet been investigated. These findings suggest that nicotine's effects in the medial prefrontal cortex and dorsal hippocampus could be mediated by a similar action. If this is the case then antagonism of nAChRs, in each of these areas, should have similar effects on trace fear conditioning. This hypothesis will be investigated in chapter 5.

Medial prefrontal cortical nicotine infusion has no effect on contextual fear conditioning

Infusion of nicotine into the medial prefrontal cortex had no effect on contextual conditioning, regardless of whether it was a component of trace or delay conditioning or whether mice were trained with 5, 2, or 1 CS-US pairings. This finding fits with literature showing that manipulations of the medial prefrontal cortex affect trace but not contextual fear conditioning. For instance, infusion of the D1 receptor antagonist SC-23390 into the medial prefrontal cortex produces deficits in trace but not contextual conditioning (Runyan & Dash, 2004), inactivation of the medial prefrontal cortex with the GABA agonist muscimol also produces deficits in trace but not contextual conditioning (Blum *et al.*, 2006), and inhibition of ERK phosphorylation in the medial prefrontal cortex prior to training produced deficits in trace but not contextual

conditioning (Runyan *et al.*, 2004). However, studies have shown that permanent and temporary lesions of the medial prefrontal cortex can produce deficits in both trace and contextual fear conditioning (Blum *et al.,* 2006; Quinn *et al.*, 2008). While it is not clear what role the medial prefrontal cortex may play in contextual conditioning, the present findings suggest that nAChRs in the medial prefrontal cortex are involved in processes that support trace but not contextual conditioning. Although, further research will be necessary to determine whether these receptors play a critical or modulatory role in trace conditioning.

No effect on delay fear conditioning

These studies show that nicotine infusion into the medial prefrontal cortex enhances trace, but not delay fear conditioning. The lack of effect of medial prefrontal cortex nicotine infusions on delay conditioning is consistent with a number of studies in eyeblink conditioning showing that lesions and inactivation of the medial prefrontal cortex produce deficits in trace, but not delay eyeblink conditioning (Kronforst-Collins & Disterhoft, 1998; McLaughlin *et al.*, 2002; Powell & Churchwell, 2002; Powell *et al.*, 2001), although, to my knowledge, the present studies are the first to demonstrate a similar dissociation in fear conditioning. Additionally, the lack of effect of medial prefrontal cortex nicotine infusion on delay conditioning is consistent with a number of studies showing that systemic nicotine administration does not affect delay fear conditioning (Gould & Higgins, 2003; Gould & Wehner, 1999; Gould *et al.*, 2004). Thus, the present studies suggest that nicotinic acetylcholinergic signaling in the medial prefrontal cortex is involved in trace but not delay fear conditioning.

Enhancement occurs at training

Lesion and inactivation studies suggest that the medial prefrontal cortex is critically involved in both acquisition and retrieval of trace fear conditioning (Blum *et al.,* 2006; Quinn *et al.*, 2008). However, dopamine receptor antagonist, ERK inhibition and protein synthesis inhibition produce deficits in trace fear conditioning if administered pre- or post-training, but do not produce deficits if administered pre-testing (Blum *et al.,* 2006; Runyan & Dash, 2004; Runyan *et al.*, 2004). Collectively, these studies suggest that while the medial prefrontal cortex is critically involved in retrieval of trace conditioning, plasticity associated events occur in the medial prefrontal cortex during or shortly after training. The present findings show that nicotine's effects in the medial prefrontal cortex on trace fear conditioning occur during training. Thus, it may be that during trace fear conditioning CS-US associations are formed in the medial prefrontal cortex, and nicotine facilitates the formation of this association.

The prelimbic cortex is a likely site of nicotine's effects on trace fear conditioning

The present studies show that nicotine infusion above or below the medial prefrontal cortex has no effect on trace fear conditioning, demonstrating that nicotine is exerting its effects within this area. The medial prefrontal cortex consists the anterior cingulate, prelimbic, and infralimbic cortices (Sesack *et al.*, 1989), and, variability in placements and estimated drug diffusion spread associated with the present studies preclude determination of whether nicotine's effects were occurring in one of these regions and not the others. However, the fact that nicotine infusion above the medial prefrontal cortex with placements near the anterior cingulate cortex, and infusion below

the medial prefrontal cortex with placements near the infralimbic cortex both had no effect on trace conditioning, suggests that nicotine's effects may have been occurring in the prelimbic cortex. Indeed, much evidence suggests critical involvement of the prelimbic cortex in trace conditioning. Single unit recording shows that in trace fear conditioning neurons in the prelimbic cortex are active during the trace interval (Gilmartin & McEchron, 2005). Immunohistological studies show that ERK phosphorylation increases in the prelimbic cortex following trace fear conditioning (Runyan & Dash, 2004; Runyan *et al.*, 2004). Additional support for the involvement of the prelimbic cortex in trace fear conditioning can be seen in its connectivity. The prelimbic cortex receives heavy afferent projections from the entorhinal cortex an area that may contribute to CS maintenance via persistent firing neurons (Bang & Brown, 2009; Hasselmo, 2006; Kholodar-Smith *et al.*, 2008), ventral hippocampus an area involved in fear expression (Esclassan *et al.*, 2009), medial dorsal thalamic nucleus an area involved in CS processing (LeDoux *et al.,* 1984; LeDoux *et al.*, 1986), and ventral tegmental nucleus a primary source of dopamine, which is involved in trace fear conditioning (Condé *et al.,* 1990; Condé *et al.*, 1995; Hoover & Vertes, 2007; Pezze & Feldon, 2004; Runyan & Dash, 2004). Additionally, analysis of the anatomical and functional connections of the medial prefrontal cortex suggest that dorsal regions (prelimbic and cingulate cortices) are involved in temporal patterning of behavioral planning, which could directly support trace conditioning, while the ventral regions (infralimbic cortex) are more involved in establishing and shifting between different responses to similar stimuli, such as during discrimination tasks and fear extinction (Heidbreder & Groenewegen, 2003). These studies suggest that the prelimbic cortex is

critically involved in trace conditioning and that this may also be the site of nicotine's effects in medial prefrontal cortex on trace fear conditioning. However, further investigation would be necessary to determine if nicotine's effects on trace fear conditioning are dependent on its action in the prelimbic cortex.

Summary

The present findings contribute to existing literature suggesting that the medial prefrontal cortex is critically involved in trace but not delay fear conditioning, as well as demonstrating that nicotine's effects in this region enhance trace but not contextual or delay conditioning. Additionally, these findings demonstrate that similar to its effects in the dorsal hippocampus, nicotine in the medial prefrontal cortex enhances acquisition of trace fear conditioning. Further, the lack of effects above or below the medial prefrontal cortex may suggest that within the medial prefrontal cortex, the prelimbic cortex is the locus of nicotine's action on trace fear conditioning. Collectively, these findings contribute to literature suggesting that the medial prefrontal cortex is critical to acquisition of trace fear conditioning, perhaps by allowing association of a CS representation and a US, and demonstrate that infusion of nicotine into this area can enhance this process. The role of the medial prefrontal cortex and nAChRs in this region will be further investigated and discussed in chapters 5 and 6.

CHAPTER 5. THE EFFECTS OF ANTAGONISM OF LOCAL HIGH-AFFINITY OR LOW-AFFINITY NICOTINIC ACETYLCHOLINERGIC RECEPTORS ON TRACE FEAR CONDITIONING

Introduction

Local nicotine infusion has different effects on trace and contextual fear conditioning depending on brain region and dose, but it is not yet clear what these effects tell us about the mechanisms that normally support trace conditioning. Infusion of nicotine into the dorsal hippocampus enhances both trace and contextual fear conditioning, although the fact that these two effects are dissociable by nicotine dose suggests that they may occur through different processes. While, infusion of nicotine into the medial prefrontal cortex produces enhancement of trace fear conditioning, without affecting contextual conditioning, suggesting that nicotinic acetylcholinergic signaling in this brain area can selectively modulate trace conditioning. In contrast to nicotine's effects in the dorsal hippocampus and medial prefrontal cortex, infusion of nicotine into the ventral hippocampus produces deficits in both trace and contextual fear conditioning. In each case these effects cannot be attributed to non-specific drug effects on anxiety or locomotion, as they do not extend to delay fear conditioning. Thus, stimulation of nAChRs has differential effects on trace and contextual fear conditioning. However, it is not clear if nAChRs in the dorsal hippocampus, medial prefrontal cortex, or ventral hippocampus support processes normally involved in trace fear conditioning or if activation of these receptors by nicotine affects conditioning through other mechanisms. If nicotinic acetylcholinergic signaling is critically involved in trace

conditioning or contextual conditioning, then decreases in nicotinic acetylcholinergic signaling in these brain regions should produce deficits, however, if nicotinic acetylcholinergic signaling modulates conditioning, but is not necessarily involved, then antagonists should have no effect.

Systemic studies suggest that nicotinic acetylcholinergic signaling is not critical to trace fear conditioning. Systemic administration of DHβE, an antagonist for high-affinity nAChRs has no effect on trace or contextual fear conditioning, although DHβE is able to precipitate withdrawal deficits in chronic nicotine treated animals, and systemic MLA, an antagonist for low-affinity nAChRs has no effect on contextual conditioning, though higher doses may produce a trend towards a deficit in trace conditioning (Portugal *et al.,* 2008; Raybuck & Gould, 2009). While, these findings suggest that nicotinic acetylcholinergic signaling may not be critical to trace conditioning, it is possible that systemic antagonism of nAChRs does not produce the same effects as antagonism in a specific brain area. In support of this multiple studies have shown that systemic and local nicotine administration differ in their effects on neural activation (Panagis *et al.,* 1996; Seppä *et al.*, 2001), neurotransmitter release (Rossi *et al.,* 2005; Shearman *et al.*, 2005; Singer *et al.*, 2004), and spatial working memory (Abdulla *et al.,* 1993; Abdulla *et al.*, 1996; Kim & Levin, 1996; Levin & Rose, 1991; Levin *et al.*, 1990; Levin *et al.*, 1994). Thus, it may be that the systemic effects of nicotine equal the sum of its local effects. Therefore, to understand how nicotine effects learning, and to understand how nicotinic acetylcholinergic signaling supports learning, we will have to investigate the role of local nicotinic acetylcholinergic signaling. Additionally, these effects may differ depending on which populations of receptors are involved.

Neuronal nicotinic receptors are pentameric ion channels, composed of different combinations of subunits. The subunit composition of nAChRs determines its response to agonists and antagonists, as well as its ion channel properties (Le Novère *et al.,* 2002; Luetje *et al.*, 1990). These receptors can be broadly grouped into two populations, those that bind to nicotine with high-affinity and those that bind to nicotine with low-affinity (Changeux & Taly, 2008; Hogg *et al.*, 2003; McGehee, 1999). High-affinity nAChRs are composed of a heteromeric combination of α and β subunits with α4β2 being expressed in the highest density in brain tissue, although expression of these heteromeric nAChRs differs by brain region as well as through development (Deutch *et al.,* 1987; Plenge & Mellerup, 1998). Alternately, low-affinity nAChRs are homogenously composed of α7 subunits, but are also heterogeneously expressed in the brain (Clarke *et al.,* 1985; Deutch *et al.*, 1987; Segal *et al.*, 1978; Ward *et al.*, 1990). These two populations of receptors have been differentially implicated in nicotine's effects and are thought to differentially mediate behavioral and cellular processes controlled by acetylcholinergic signaling (Kenney & Gould, 2008; Levin *et al.*, 2006; Patrick *et al.*, 1993; Paylor *et al.*, 1998; Picciotto *et al.*, 1995). Thus, it may be that in different brain regions, different populations of nAChRs are involved in trace fear conditioning.

Scientific Questions (Design)

As nicotine infusion into the dorsal hippocampus, ventral hippocampus, and medial prefrontal cortex all affect trace fear conditioning, it is possible that endogenous acetylcholinergic signaling through nicotinic receptors is a critical mediator of processes supporting trace fear conditioning in these brain areas. Additionally, it may be the case

that a role for acetylcholinergic signaling is mediated by either high- or low-affinity nAChRs. Further, it is possible that involvement of nicotinic acetylcholinergic signaling in this task is different at training, where it would be mediating processes supporting acquisition or consolidation of the trace conditioned CS-US relationship, as opposed to at testing where it would be mediating processes supporting retrieval of the CS-US association or fear expression. Thus, to determine whether high-affinity or low-affinity nicotinic acetylcholinergic signaling in the dorsal hippocampus, ventral hippocampus, or medial prefrontal cortex is critically involved in trace fear conditioning, the following studies examined the effects of the high-affinity antagonist DHβE and the low-affinity antagonist MLA on trace fear conditioning. Additionally, effective doses of antagonists were administered into these brain areas at training or testing, to determine if they were affecting processes involved in acquisition or expression of trace conditioning.

Methods

Subjects

These experiments used 271 8-12 week old male C57BL/6J mice. All mice were singly housed in standard colony cages, maintained on a 12h light/dark cycle with lights on at 7:00 am, and allowed *ad libitum* access to food and water. Housing, surgical, and behavioral procedures were approved by the Temple University Animal Care and Use Committee, and were in accordance with ethical standards of the APA.

Materials

Drugs and Infusion

Dihydro-beta-erythroidine (DHβE) 4.5, 9, & 18.00 μg/side, and methyllycaconitine 6.75, 13.5, 27 μg/side were obtained from Sigma-Aldrich (St. Louis, MO). Drugs were directly infused through 22-gauge (dorsal and ventral hippocampus) or 33-gauge (medial prefrontal cortex) cannula. During infusions mice were gently restrained, while stainless steel stylets were removed from guide cannula and replaced with infusion cannula. Drugs were infused at a rate of 0.50 μl/min and at an injection volume of 0.50 μl per side. Infusion cannula were attached to polyethylene tubing (PE50; Plastics One) attached to a 10 μl Hamilton syringe (Reno, NV) connected to a microinfusion pump (KDS 100; KD Scientific, New Hope, PA). Infusion cannula were left in place for 30 seconds after infusion. DHβE and MLA were infused 25 min before training and/or testing. Spread of infusion using this procedure has been previously estimated to be ~1mm^3 (Lewis & Gould, 2007).

Apparatus

Training was conducted in conditioning chambers (model 307AW, Med Associates, St. Albans, VT) housed in sound attenuating cubicles. An 85 dB white noise conditioned stimulus (CS) was administered through speakers attached to the right wall of each chamber. 69 dB background noise was provided by 50 mm ventilation fans, mounted on the right wall of each sound-attenuating cubicle. A 2 second, 0.57 mA footshock unconditioned stimulus (US) was administered through the chamber floors, which were composed of 18 stainless steel bars and connected to a shock generator and

scrambler (Med-Associates). Stimulus administration was controlled by an IBM-compatible PC running Med-PC software (Med-Associates).

Testing of trace fear conditioning was conducted in four conditioning chambers situated in sound attenuating cubicles located in a different room than that used for training. The testing chambers were distinct from the training chambers and had white plastic floors, stainless steel sides, and Plexiglas panels for the front, rear and lid. Additionally, a novel olfactory cue (artificial vanilla extract) was applied to paper toweling placed below each of the chamber floors. Ventilation fans mounted on the right wall of the sound attenuating cubicles will provide background noise. CS was generated with a Grason-Stradler noise generator (model 901B, West Concord, MA) attached to 3-inch speakers mounted on the left side of each of the conditioning chambers.

Procedure

Surgical

For surgical procedures mice were anesthetized with isoflurane, and placed in a mouse stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). The scalp was shaved, scrubbed and retracted to expose the skull. Holes were drilled for anterior and lateral coordinates, determined from Paxinos and Franklin (2001), guide cannula were lowered into place with the stereotaxic, to match D/V coordinates, and permanently secured with dental cement. Dorsal hippocampal coordinates were A/P – 1.7, M/L 1.5, D/V 2.3; ventral hippocampal coordinates were A/P –2.8, M/L 3.0, D/V 4.0; and medial prefrontal cortical coordinates were A/P 1.7, M/L 1.0, D/V 2.5, relative to bregma. All cannula were purchased from Plastics One (Roanoke, Virginia). Stainless steel stylets

were placed in the guide cannula to maintain patency during the 5-day recovery period. All animals received postoperative analgesic/anti-inflammatory ketprofen (2 mg/kg, sc; Fort Dodge, Fort Dodge, IA).

Behavioral

Behavioral procedures were based on previous studies (Davis & Gould, 2007; Gould *et al.*, 2004). Training of trace fear conditioning was conducted during a single 16-minute training session wherein the mice were presented 5 CS-US pairings separated by a variable inter-trial-interval (90-120 seconds). CS-US parings consisted of a 30 second, 85 dB white noise CS presentation, followed by a 30 second trace interval, and terminated with the presentation of a 2 second, 0.57 mA footshock US. The training session began with activation of the house light and terminated 30 seconds following the last US presentation, at which point the house light was extinguished and mice were placed in their home cage. Twenty-four hours following training, mice were tested for both contextual and trace-cued associative conditioning. To test for contextual associations formed during the training session mice were placed in the training context and observed for freezing for five minutes. One to two hours later, to test for trace-cued conditioning, mice were placed in the altered testing chambers for six minutes, for the first three minutes no CS was presented and mice were scored for freezing to the altered context, a measure of generalized fear, then the CS was presented for three minutes and mice were scored for trace-cued freezing.

Histology

All brains were post-fixed in formalin for at least 24 hours, sliced on a cryostat, mounted, and nissl stained. Infusion coordinates were confirmed with either dye infusion, or by observing gliosis along the infusion cannula tracts.

Analysis

Analysis was conducted with SPSS 17. Data were analyzed with one-way ANOVA followed with Tukey's HSD post hoc tests. Data sets not meeting homogeneity of variance assumption of the ANOVA were followed up with a Games-Howell post-hoc test.

Results

Dorsal Hippocampus

Placements

Histological analysis of dorsal hippocampal infusions showed that out of 87 surgical cannula implantations none were outside of the target area. Diagrams of placements for each study are displayed with the behavioral data.

DHβE

Infusion of nicotine into the dorsal hippocampus enhances both trace and contextual fear conditioning, see chapter 2 and (Davis *et al.*, 2007). While these data

demonstrate that nicotinic receptors in the dorsal hippocampus can modulate both trace and contextual conditioning, it is not clear whether nicotinic acetylcholinergic signaling in the dorsal hippocampus is critical to trace and contextual fear conditioning. Thus, to determine if nicotinic acetylcholinergic signaling in the dorsal hippocampus is critical to trace and contextual fear conditioning, I infused DHβE, an antagonist for high-affinity nAChRs into the dorsal hippocampus prior to training and testing of trace fear conditioning. ANOVA showed that infusion of DHβE into the dorsal hippocampus significantly affected trace [$F(3,27) = 30.927$, $p < 0.000$] conditioning, but had no effect on contextual conditioning or baseline or altered freezing, Figure 21. Post hoc analysis revealed that mice treated with 4.5 or 9 μg/side DHβE showed deficits in trace conditioning compared to saline treated controls, but that mice treated with 18 μg/side DHβE were no different than controls.

Figure 21.

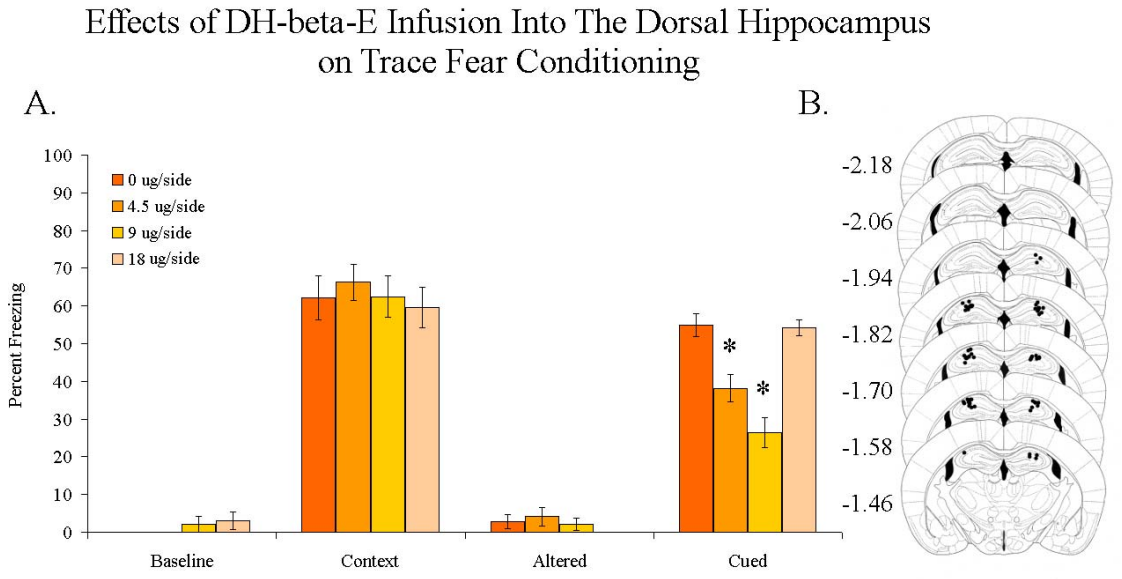

Figure 21: Antagonism of high-affinity nAChRs in the dorsal hippocampus produces dose-dependent deficits in trace fear conditioning. A. **DHβE (4.5 & 9 µg/side) produces deficit in trace but not contextual fear conditioning, suggesting that high-affinity nicotinic acetylcholinergic signaling in the dorsal hippocampus supports trace conditioning. Significant difference ($p < 0.05$) from saline treated control group denoted with (*) data are reported as mean ± standard error of the mean. Subjects per group were 8 (0.0, 4.5, 9.0, 18.0 µg) and 7 (9.0 µg).** B. **Histological analysis confirmed that all infusions were directed into the dorsal hippocampus.**

DHβE- Training or Testing

Infusion of the high-affinity nAChR antagonist DHβE into the dorsal hippocampus produced deficits in trace conditioning. As the dorsal hippocampus is critically involved in both acquisition and expression of recent trace conditioning (Quinn *et al.*, 2008), it is possible that actions of DHβE on trace conditioning could reflect mediation of either acquisition or retrieval by high-affinity nAChRs in the dorsal hippocampus. Thus, to determine if trace conditioning involves high-affinity nAChRs at training or at testing, I infused DHβE (9 µg/side) at either training or testing of trace fear

conditioning. ANOVA showed that infusion of DHβE into the dorsal hippocampus had a significant effect on trace [F (2,23) = 42.171, $p < 0.000$] but not contextual conditioning or baseline or altered freezing, Figure 22. Post hoc analysis demonstrated that administration of DHβE into the dorsal hippocampus prior to training produced significant deficits in trace conditioning, but administration prior to testing had no effect. Thus, it is likely that activation of high-affinity nAChRs in the dorsal hippocampus mediate the acquisition of trace fear conditioning.

Figure 22.

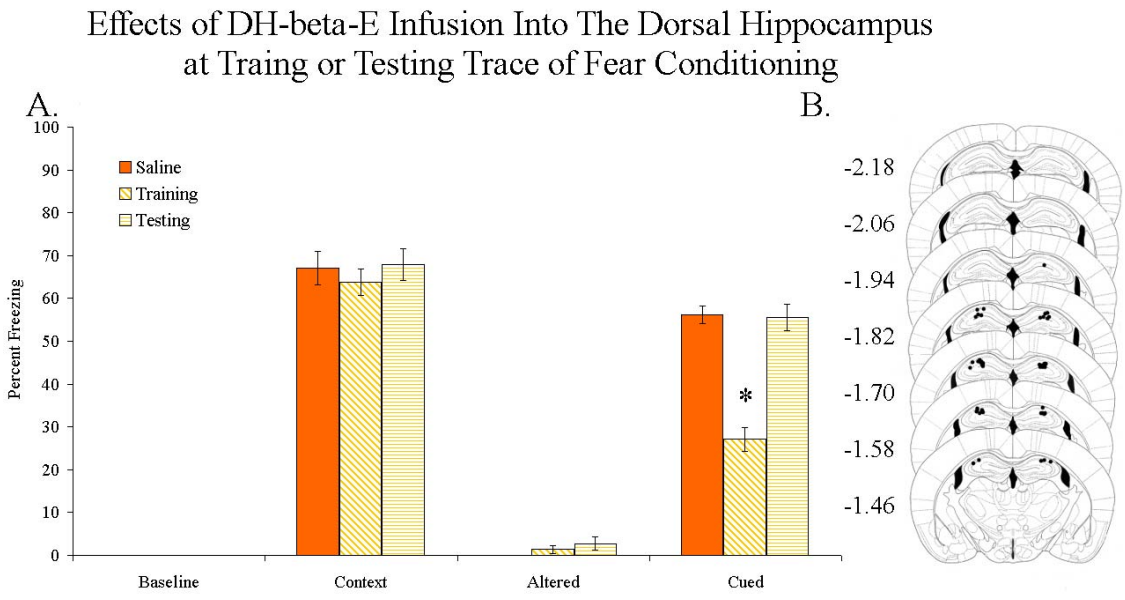

Figure 22: High-affinity nAChR activity in the dorsal hippocampus is critical for acquisition of trace fear conditioning. A. **Infusion of DHβE (9 µg/side) into the dorsal hippocampus at training produced deficits in trace conditioning, while infusion prior to testing had no effect. These results suggest that high-affinity nAChR activity in the dorsal hippocampus is involved in the acquisition of the trace conditioned CS-US association. Significant difference ($p < 0.05$) from control group denoted with (*) data are reported as mean ± standard error of the mean. There were 8 subjects per group.** B. **Histological analysis confirmed that all cannula placements were directed into the dorsal hippocampus.**

MLA

Acquisition of trace fear conditioning involves high-affinity nAChR signaling in the dorsal hippocampus. However, it is possible that low-affinity nAChRs in this brain area are also involved. To determine if low-affinity nAChRs in the dorsal hippocampus are involved in trace fear conditioning, I infused the low-affinity nAChR antagonist MLA across a range of doses prior to training and testing of trace fear conditioning. ANOVA showed that infusion of MLA at 6.75, 13.5, or 27 µg/side had no effect on trace or contextual conditioning, or baseline or altered freezing, Figure 23.

Figure 23.

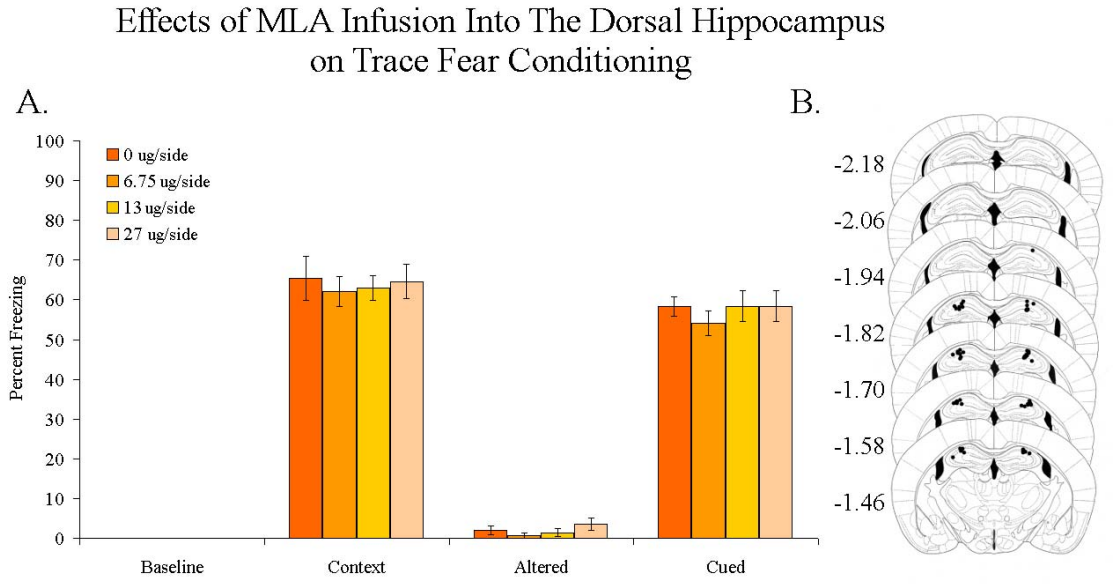

Figure 23: Low-affinity nAChRs in the dorsal hippocampus are not involved in trace fear conditioning. A. **Infusion of MLA into the dorsal hippocampus had no effect on trace or contextual fear conditioning, suggesting that these receptors are not involved in the actions of the dorsal hippocampus during fear conditioning. There were 8 subjects per group.** B. **Histological analysis confirmed that all infusions were directed into the dorsal hippocampus.**

Ventral Hippocampus

Placements

Histological analysis of all ventral hippocampal placements showed that out of 63 cannula implantations none were outside of the target area. Diagrams of placements for each study are displayed with the behavioral data.

DHBE

Nicotine infusion into the ventral hippocampus produces deficits in both trace and contextual fear conditioning. It is not known if these effects represent alteration of ventral hippocampal processes normally involved in trace and contextual fear conditioning. Thus, to determine if trace and contextual fear conditioning depend upon high-affinity nAChR signaling in the ventral hippocampus, I infused the high-affinity nAChR antagonist DHβE across a range of doses into the ventral hippocampus prior to training and testing of trace fear conditioning. ANOVA showed that infusion of DHβE into the ventral hippocampus at 4.5, 9 or 18 μg/side had no effect on contextual or trace conditioning, or on baseline or altered freezing, Figure 24.

Figure 24.

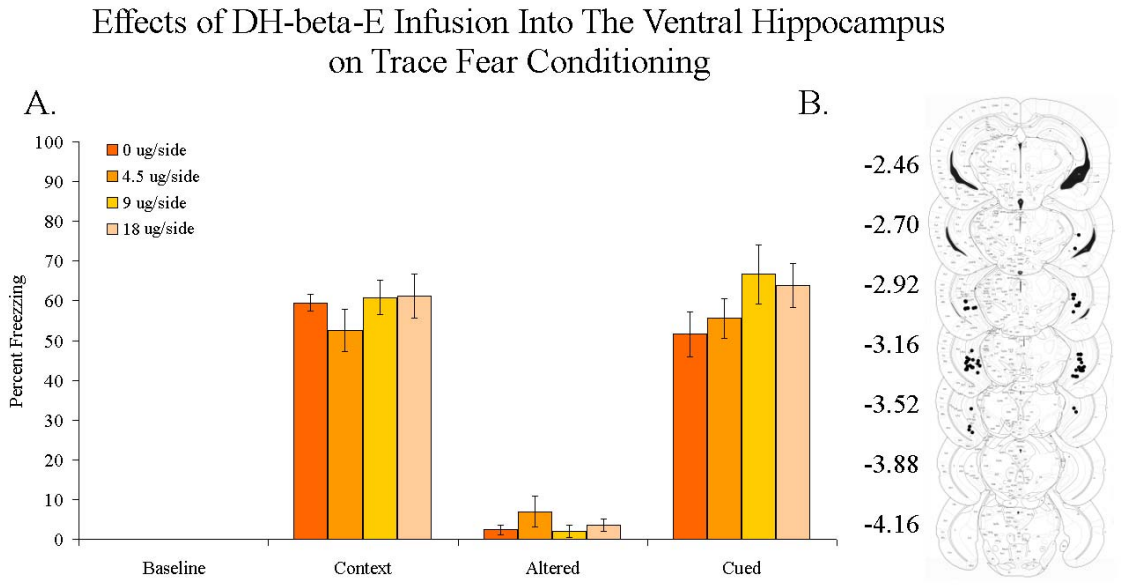

Figure 24: High-affinity nAChR signaling in the ventral hippocampus is not critically involved in trace fear conditioning. A. **Infusion of DHβE into the ventral hippocampus had no effect on trace or contextual fear conditioning, suggesting that these receptors may not be critically involved in fear conditioning. Subjects per group were 7 (0.0 µg) and 8 (4.5, 9.0, 18.0 µg).** B. **Histological analysis confirmed that all infusions were directed in to the ventral hippocampus.**

MLA

Antagonism of high-affinity nAChRs in the ventral hippocampus had no effect on trace fear conditioning, but it is possible that low-affinity nicotinic receptors are involved in this task. Thus, to determine if trace and contextual fear conditioning depend upon low-affinity nAChR signaling in the ventral hippocampus, I infused the low-affinity nAChR antagonist MLA across a range of doses into the ventral hippocampus prior to training and testing of trace fear conditioning. ANOVA showed that infusion of MLA into the ventral hippocampus at 6.75, 13.5 or 27 µg/side had no effect on contextual or trace conditioning, or on baseline or altered freezing, Figure 25.

Figure 25.

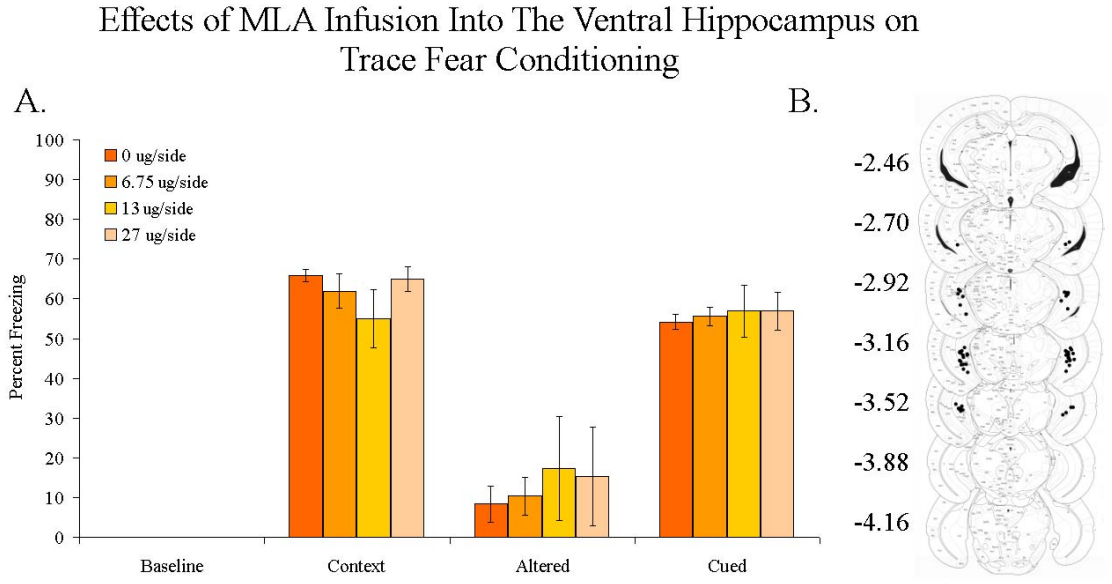

Figure 25: Low-affinity nAChR signaling in the ventral hippocampus is not critically involved in trace fear conditioning. A. **Infusion of MLA into the ventral hippocampus had no effect on trace or contextual fear conditioning. Thus, it is unlikely that low-affinity nAChRs in the ventral hippocampus are critically involved in fear conditioning. There were 8 subjects per group.** B. **Histological analysis confirmed that all infusions were directed into the ventral hippocampus.**

Medial Prefrontal Cortex

Placements

Histological analysis of all medial prefrontal cortical placements showed that out of 121 surgical cannula implantations none were outside of the target area. Diagrams of placements for each study are displayed with the behavioral data.

DHBE

Infusion of nicotine into the medial prefrontal cortex selectively enhanced trace fear conditioning. It is not yet clear whether this reflects a critical involvement of

nAChRs in the medial prefrontal cortex in trace conditioning, or if it demonstrates the ability of nAChR signaling to modulate trace fear conditioning. Thus, I infused DHβE into the medial prefrontal cortex at a range of doses prior to training and testing of trace fear conditioning. ANOVA showed that infusion of DHβE affected trace [$F(3,30) = 11.149$, $p < 0.000$] conditioning, but had no effect on contextual conditioning, or baseline or altered freezing, Figure 26. Post hoc analysis revealed that DHβE at 9 or 18 µg/side enhanced trace conditioning.

Figure 26.

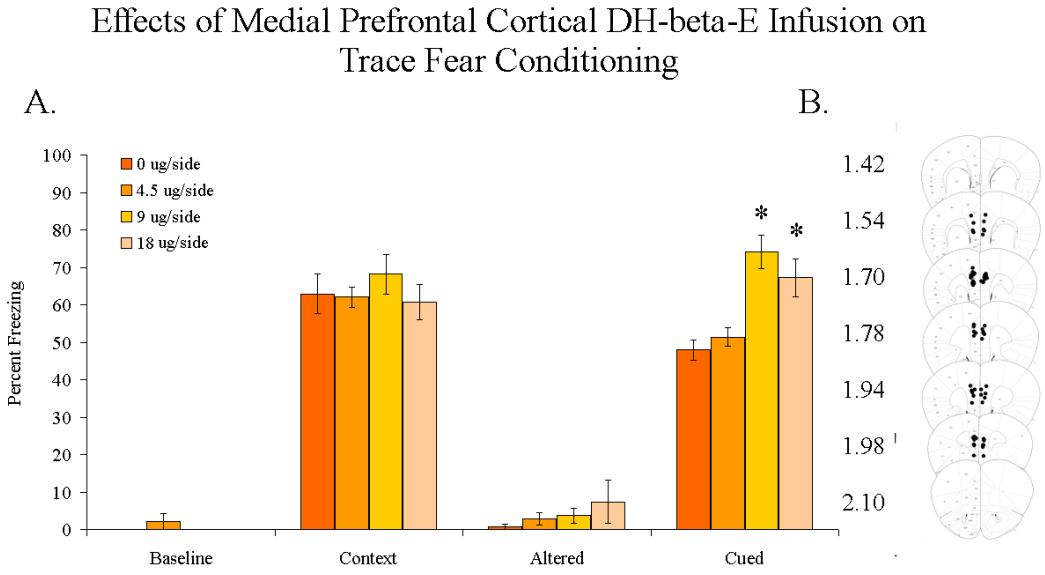

Figure 26: Antagonism of high-affinity nAChRs in the medial prefrontal cortex enhances trace fear conditioning. A. **Infusion of DHβE into the medial prefrontal cortex dose-dependently enhanced trace fear conditioning but did not affect contextual conditioning. This suggests that decreases in high-affinity nAChR signaling in the medial prefrontal cortex modulate trace conditioning. Significant difference ($p < 0.05$) from saline treated control group denoted with (*) data are reported as mean ± standard error of the mean. Subjects per group were 8 (0.0, 4.5 µg) and 9 (9.0, 18.0 µg).** B. **Histological analysis confirmed that all infusions were directed into the medial prefrontal cortex.**

DHBE- Training or Testing

Infusion of the high-affinity nAChR antagonist DHβE into the medial prefrontal cortex produced dose-dependent enhancement of trace fear conditioning. To determine if this effect was involved in acquisition of the CS-US association at training or involved in retrieval of the trace-conditioned memory at testing, I administered DHβE (9 μg/side) at either training or testing of trace fear conditioning. ANOVA showed that infusion of DHβE into the medial prefrontal cortex at training or testing of trace fear conditioning affected both contextual [$F(2,23) = 7.548$, $p < 0.003$] and trace conditioning [$F(2,23) = 29.056$, $p < 0.000$], but had no effect on baseline or altered freezing. Post hoc analysis revealed that DHβE infusion at training produced significant enhancement of trace conditioning, without affecting contextual conditioning, suggesting that enhancement of trace fear conditioning by DHβE in the medial prefrontal cortex occurs at training of trace conditioning, potentially by altering processes underlying acquisition of the trace-conditioned CS-US relationship. Alternately, post hoc analysis showed that administration of DHβE into the medial prefrontal cortex prior to testing produced deficits in both contextual and trace fear conditioning, but did not affect baseline or altered freezing, Figure 27. This surprising finding suggests that the medial prefrontal cortex is able to modulate fear expression and that high-affinity nicotinic acetylcholinergic signaling in this brain region may have a role in this process.

Figure 27.

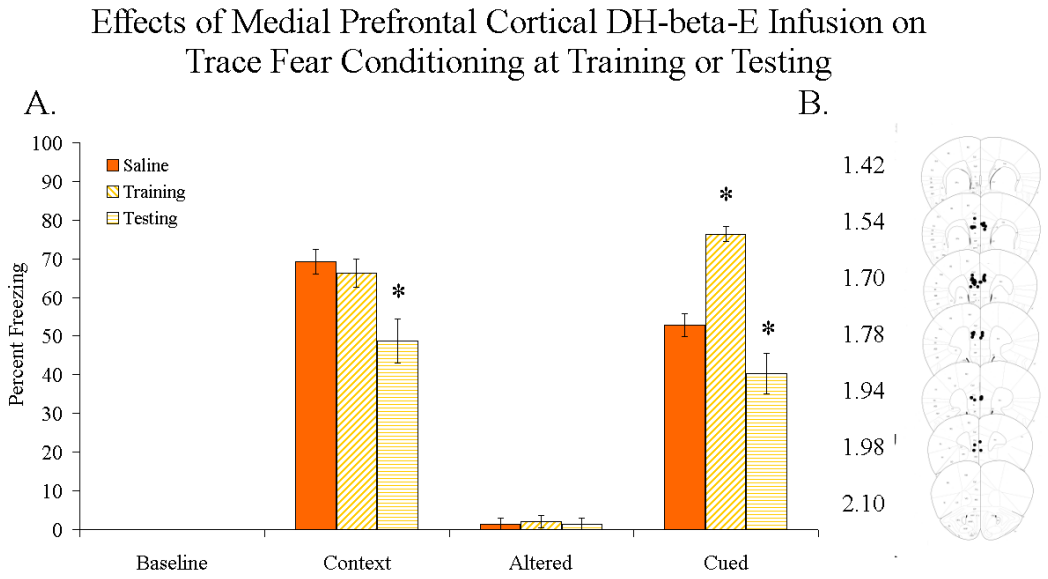

Figure 27: Antagonism of high-affinity nAChRs in the medial prefrontal cortex alters trace fear conditioning. A. **Infusion of DHβE (9 µg/side) into the medial prefrontal cortex enhances trace conditioning, but paradoxically DHβE infusion at testing produces deficits in both trace and contextual conditioning. These results suggest that high-affinity nAChR in the medial prefrontal cortex modulate acquisition of trace conditioning, and that this receptor population can also modulate retrieval of hippocampus-dependent learning. Significant difference ($p < 0.05$) from saline treated control group denoted with (*) data are reported as mean ± standard error of the mean. There were 8 subjects per group.** B. **Histological analysis confirmed that all infusions were directed into the medial prefrontal cortex.**

MLA

Infusion of DHβE into the medial prefrontal cortex enhances trace fear conditioning, as does nicotine infusion. While this finding suggests that nicotine's effects on trace fear conditioning in the medial prefrontal cortex may be mediated by desensitization of high-affinity nicotinic receptors, it is not clear these are the only nAChRs involved. Thus, to determine if low-affinity nAChRs in the medial prefrontal cortex are also involved in trace fear conditioning, I infused the low-affinity nAChR

antagonist MLA into the medial prefrontal cortex at a range of doses prior to training and testing of trace conditioning. ANOVA showed that infusion of MLA affected trace [$F(3,35) = 4.993$, $p < 0.005$] conditioning but had no effect on contextual conditioning, and enhanced altered [$F(3,35) = 5.407$, $p < 0.004$] freezing but did not affect baseline freezing. Post hoc analysis showed that the two higher doses of MLA 13.5 and 27 µg/side enhanced trace conditioning, while the lower dose 6.75 µg/side enhanced altered freezing, Figure 28. As these two effects occur at different doses, it seem unlikely that MLA infusion into the medial prefrontal cortex enhances trace conditioning by enhancing generalized freezing.

Figure 28.

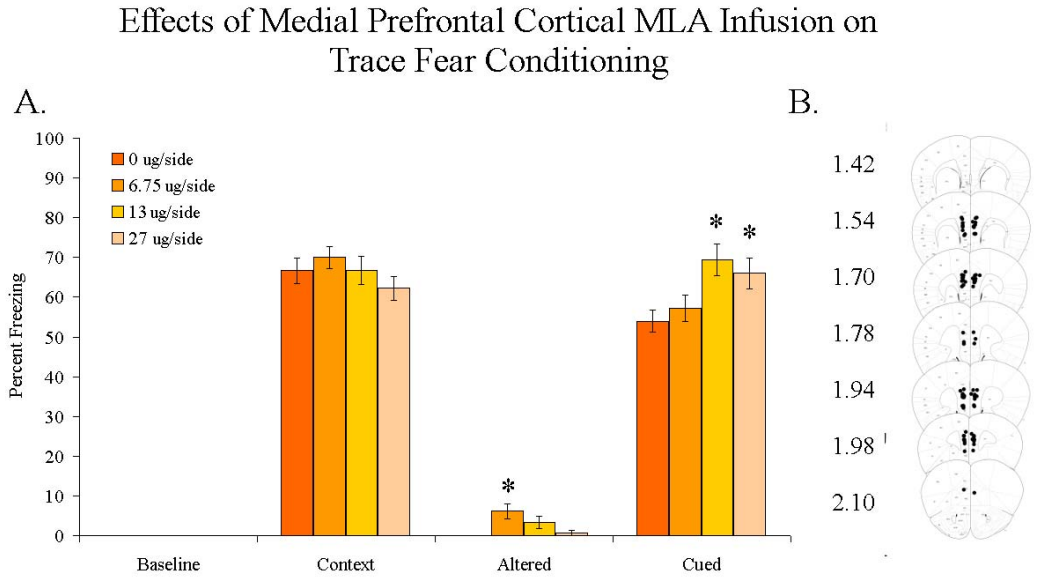

Figure 28: Antagonism of low-affinity nAChRs in the medial prefrontal cortex enhances trace fear conditioning. A. **Infusion of MLA into the medial prefrontal cortex dose-dependently enhanced trace fear conditioning but did not affect contextual conditioning. This suggests that decreases in low-affinity nAChR signaling in the medial prefrontal cortex can enhance trace conditioning. Significant difference ($p < 0.05$) from saline treated control group denoted with (*) data are reported as mean ± standard error of the mean. Subjects per group were 10 (0.0, 6.75, 13.5 µg) and 9 (27.0 µg).** B. **Histological analysis confirmed that all infusions were directed into the medial prefrontal cortex.**

MLA- Training or Testing

Infusion of the low-affinity nAChR antagonist MLA into the medial prefrontal cortex produced dose dependent enhancement of trace fear conditioning. To determine if this effect was mediated by alteration of processes supporting acquisition or expression of the trace conditioned CS-US association, I administered MLA (13.5 μg/side) into the medial prefrontal cortex at training or testing of trace fear conditioning. ANOVA showed that MLA infusion at either training or testing had a significant effect on both contextual [$F(2,21) = 38.425$, $p < 0.000$] and trace conditioning [$F(2,21) = 26.808$, $p < 0.000$], but had no effect on baseline or altered freezing, Figure 29. Post hoc analysis revealed that MLA infusion at training significantly enhanced trace conditioning, without affecting contextual conditioning, suggesting that enhancing effects of MLA infusion into the medial prefrontal cortex occur at training, likely by enhancing processes related to acquisition of the trace conditioned CS-US association. Alternately, infusion of MLA into the medial prefrontal cortex at testing produced significant deficits in both contextual and trace conditioning. This surprising finding may suggest that low-affinity nAChRs in the medial prefrontal cortex can modulate expression of fear conditioning.

Figure 29.

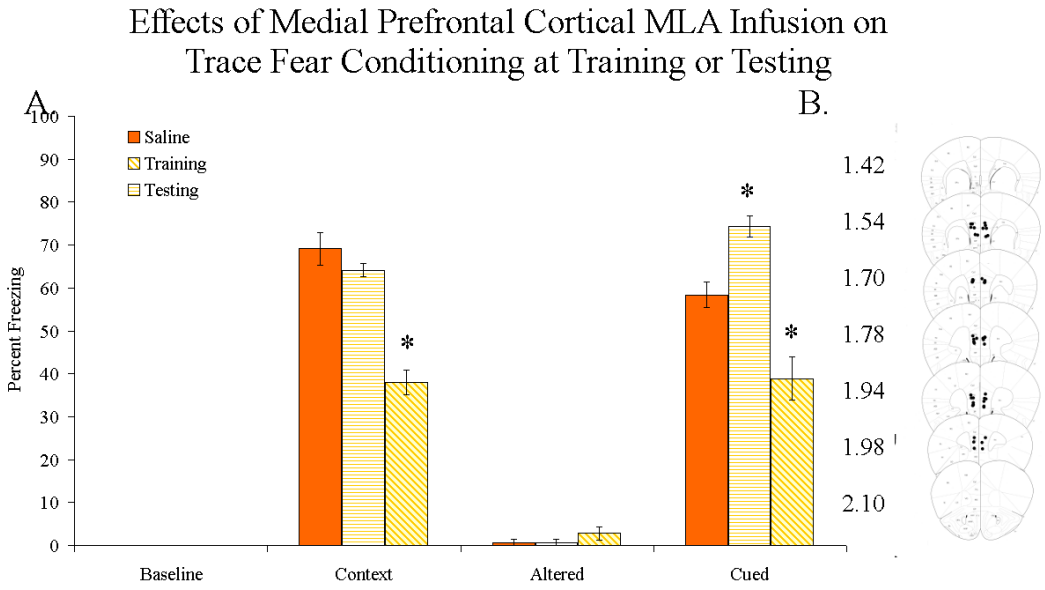

Figure 29: Antagonism of low-affinity nAChRs in the medial prefrontal cortex alters trace fear conditioning. A. **Infusion of MLA (13.5 µg/side) into the medial prefrontal cortex at training enhances trace conditioning, but paradoxically MLA infusion at testing produces deficits in both trace and contextual conditioning. These results show that low-affinity nAChR antagonist in the medial prefrontal cortex can enhance acquisition of trace conditioning, but also that antagonism of this receptor population can also produce deficits in retrieval of hippocampus-dependent learning. Significant difference ($p < 0.05$) from saline treated control group denoted with (*) data are reported as mean ± standard error of the mean. There were 8 subjects per group.** B. **Histological analysis confirmed that all cannula placements were directed in to the medial prefrontal cortex.**

Conclusions

Dorsal hippocampus

The present findings demonstrate that high-affinity nAChR signaling in the dorsal hippocampus supports trace fear conditioning but not contextual fear conditioning and that this effect occurs at training on processes related to acquisition of the CS-US association. This is consistent with findings from chapter 2 showing that nicotine infusion into the dorsal hippocampus enhances acquisition of trace fear conditioning. However, these findings do not fit with the effects of systemic DHβE administration on

trace fear conditioning, where no deficits were seen, or with the effects of systemic MLA on trace fear conditioning, where a trend towards a deficit was seen at higher doses (Raybuck & Gould, 2009). Although, the present findings do fit with multiple reports that systemic and local DHβE and MLA have no effect on contextual fear conditioning (Davis & Gould, 2006; Davis *et al.*, 2007; Portugal *et al.*, 2008; Raybuck & Gould, 2009). Thus, these findings suggest that processes mediated by high-affinity nAChRs in the dorsal hippocampus support acquisition of trace conditioning, that nicotinic effects on trace and contextual conditioning are mediated by different mechanisms, and that systemic antagonism of high-affinity nAChRs is not equal to local antagonism of high-affinity nAChRs. This conclusion in consistent with studies suggesting that different processes in the dorsal hippocampus are involved in trace and contextual conditioning (Quinn *et al.,* 2005; Weitemier & Ryabinin, 2004). These points will be further discussed in chapter 6.

Ventral Hippocampus

Infusion of nicotinic antagonists into the ventral hippocampus had no effect on trace or contextual fear conditioning, although studies in Chapter 3 demonstrate that activation of nicotinic receptors in the ventral hippocampus produces deficits in both trace and contextual conditioning. These effects fit with the systemic effects of nicotinic antagonists on fear conditioning, where no effects are seen (Davis & Gould, 2006; Davis *et al.*, 2007; Portugal *et al.*, 2008; Raybuck & Gould, 2009), although systemic administration of MLA produced a trend toward a deficit in trace fear conditioning (Raybuck & Gould, 2009). In Chapter 4 it is discussed that nicotine's effect on trace and

contextual fear conditioning in the ventral hippocampus may be the result of suppression of information flow from the dorsal hippocampus to the amygdala. The present findings suggest that while nAChRs in the ventral hippocampus may be able to suppress trace and contextual conditioning, this is not a process that normally occurs during conditioning, otherwise antagonism of these receptors would alter information flow through the ventral hippocampus, producing changes in conditioning. It is possible that such a nAChR-mediated mechanism could support suppression of fear expression to contextual and trace conditioned stimuli under other circumstances, such as during extinction; further discussion of this topic is provided in Chapter 6.

Medial Prefrontal Cortex

Findings from Chapter 4 demonstrate that infusion of nicotine into the medial prefrontal cortex enhances acquisition of trace fear conditioning, but the present findings show that infusion of antagonists into the medial prefrontal cortex has the same effect, suggesting that the mechanism through which nicotine affects trace conditioning in the medial prefrontal cortex may not be activation, but rather desensitization of nAChRs. Indeed, activation and desensitization are both thought to drive the behavioral effects of nicotine (Picciotto *et al.*, 2008). Additionally, the present findings that medial prefrontal cortical infusion of both nicotine and nicotinic antagonists modulates fear conditioning suggest it is likely that endogenous acetylcholinergic signaling participates in the acquisition of trace fear conditioning, this role will be further discussed in Chapter 6.

One surprising finding is that administration of nicotinic antagonists at training enhances trace conditioning without affecting contextual conditioning, but administration

at testing produces deficits in both trace and contextual conditioning. This finding may suggest that the effects of nicotine and nicotinic antagonists occur through different mechanisms, as both manipulations cause selective enhancement of trace conditioning during training, but nicotine has no effect at testing, and antagonists produce deficits in both trace and contextual conditioning at testing. This effect is difficult to explain, but one difference between desensitization of nAChRs by nicotine and antagonism by DHβE or MLA is that nicotine desensitizes both receptor populations (Booker *et al.,* 1998; Dani *et al.*, 2000; Fenster *et al.*, 1997; James *et al.*, 1994; Marks *et al.*, 1994; Ochoa *et al.*, 1990; Picciotto *et al.*, 2008; Pidoplichko *et al.*, 1997; Vibat *et al.*, 1995; Wang & Sun, 2005) albeit with different affinities, whereas, DHβE preferentially antagonizes high-affinity receptors (Harvey & Luetje, 1996; Harvey *et al.*, 1996; Williams & Robinson, 1984), and MLA preferentially antagonizes low-affinity receptors (Alkondon & Albuquerque, 1993; Macallan *et al.*, 1988; Ward *et al.*, 1990). Indeed, previous studies suggest that antagonism of different classes of nicotinic receptors can have effects that are subtractive rather than additive. For instance, infusion of MLA or DHβE into the dorsal hippocampus or the basolateral amygdala produces deficits in spatial working memory, however, in each area, co-administration of these antagonists had less of an effect than administration of either antagonist alone, suggesting that conjoint antagonism of high- and low-affinity nAChRs may be less detrimental than antagonism of either receptor population alone (Addy *et al.,* 2003; Nott & Levin, 2006). Additionally high- and low-affinity nAChRs each modulate different aspects of dopamine release (Ungless & Cragg, 2006), a process in the medial prefrontal cortex that is critical to trace conditioning (Runyan & Dash, 2004), which is discussed in Chapter 6 as a potential

mechanisms through which nicotine could modulate trace fear conditioning. Thus, it may be that different effects of nicotine and antagonist infusion at testing of trace fear conditioning occur because nicotine desensitizes both receptor populations, inhibiting all nicotinic acetylcholinergic signaling, whereas the antagonists selectively inhibit signaling through one population or the other. If this is the case, then it could be expected that an antagonist for both populations of receptors would not produce retrieval deficits.

Summary

These findings demonstrate that high-affinity nicotinic acetylcholinergic signaling in the dorsal hippocampus is critical to acquisition of trace conditioning, that endogenous nicotinic acetylcholinergic signaling in the ventral hippocampus does not support trace fear conditioning, and that alteration of nicotinic acetylcholinergic signaling in the medial prefrontal cortex has differential effects on conditioning, with antagonist infusion at training producing enhancements similar to those produced by nicotine and antagonist administration at testing producing deficits in expression of both trace and contextual fear conditioning. Collectively, these results suggest that endogenous nicotinic acetylcholinergic signaling is involved in trace fear conditioning, but that this involvement differs by brain region.

CHAPTER 6. A ROLE FOR NICOTINIC ACETYLCHOLINERGIC SIGNALING IN TRACE FEAR CONDITIONING

Major Findings

Findings from chapters 2 through 5 (See Table 1) demonstrate that nicotine and nicotinic antagonists in the dorsal hippocampus, ventral hippocampus, and medial prefrontal cortex have different effects on trace and contextual fear conditioning. In the dorsal hippocampus, activation of nAChRs by nicotine enhances acquisition of both trace and contextual conditioning. Although, these two effects are dissociated by dose in a 2-pairing trace fear conditioning paradigm. Additionally, antagonism of high-affinity nAChRs in the dorsal hippocampus produces deficits in acquisition of trace but not contextual fear conditioning, Table 1. These striking findings suggest that nicotine's effects in the dorsal hippocampus on trace and contextual conditioning occur through different mechanisms. A model for the role of the dorsal hippocampus and the implications of these findings for trace fear conditioning will be discussed later. In the ventral hippocampus nicotine produces deficits in both trace and contextual conditioning, Table 1. These effects occur at either training or testing, but do not extend to delay conditioning, suggesting that these deficits are specific to acquisition and retrieval of trace and contextual fear conditioning. Additionally, lack of effect of nicotinic antagonists on trace or contextual fear conditioning suggests that activation of nAChRs in the ventral hippocampus is not a critical component of trace or contextual fear conditioning. A potential role for nAChRs in the ventral hippocampus in fear conditioning will be discussed later. In the medial prefrontal cortex stimulation of nAChRs by nicotine selectively enhances acquisition of trace fear conditioning.

Surprisingly, nicotinic antagonists also selectively enhance acquisition of trace fear conditioning, Table 1. These striking findings suggest that nAChRs in the medial prefrontal cortex are involved in trace fear conditioning and that nicotine may act in the medial prefrontal cortex through receptor desensitization. Collectively, these studies demonstrate a role for nicotinic signaling in modulation and support of trace fear conditioning, demonstrate that these brain areas have different roles in supporting trace fear conditioning, and demonstrate that different processes mediate the effects of nicotine on trace and contextual fear conditioning.

Table 1.

		Protocol							
		Trace (5-pr)		Trace (2-pr)		Delay (2-pr)		Delay (1-pr)	
		Effective Dose		Effective Dose		Effective Dose		Effective Dose	
Region	**Drug**	Context	Cued	Context	Cued	Context	Cued	Context	Cued
Dorsal Hippocampus	Nicotine	tr 0.09 ▲	tr 0.09 ▲	0.36 ▲	0.09 ▲	0.36 ▲	-	-	-
	DHbE	tr 4.5 ▼	-						
	MLA	-	-						
Ventral Hippocampus	Nicotine	tr,t 0.36 ▼	tr,t 0.36 ▼	0.36 ▼	0.36 ▼	0.36 ▼	-	0.36 ▼	-
	DHbE	-	-						
	MLA	-	-						
Medial Prefrontal Cortex	Nicotine	-	tr 0.09 ▲	-	0.09 ▲	-	-	-	-
	DHbE	t 4.5 ▼	tr 4.5 ▲, t ▼						
	MLA	t 13.5 ▼	tr 13.5 ▲, t ▼						
			Effective Dose in (ug/side)			- denote no effect			
			▲ denotes enhancement			tr denote effect at training			
			▼ denote deficit			t denotes effect at testing			

Table 1: Summary of findings from Chapters 2 through 5.

A model of the circuitry supporting nicotine's effect on trace fear conditioning.

The present findings along with previous reports suggest that multiple brain regions act in concert to allow acquisition of the trace-conditioned CS-US association.

The role of the dorsal hippocampus, ventral hippocampus, and medial prefrontal cortex in trace fear conditioning as well as the effects of nicotine in each of these regions can be described as part of a learning circuit in which each region has a distinct function, Figure 30.

Role of the dorsal hippocampus in trace fear conditioning

The dorsal hippocampus may support trace fear conditioning by maintaining a neural representation of the CS during the trace interval, see Figure 30. Indeed, dorsal hippocampal involvement depends upon the length of trace interval (Burman & Gewirtz, 2007; Chowdhury *et al.*, 2005; Misane *et al.*, 2005), suggesting that ability to maintain the CS is increasingly dependent on the dorsal hippocampus with longer trace intervals. Additionally, the present findings that nicotine's effects on trace fear conditioning occur during acquisition, and that the dorsal hippocampus is not necessary for retrieval of remote memories for trace conditioning (Quinn *et al.*, 2008), suggest that this brain region is more involved in acquisition than retrieval of trace conditioning,. Further, computational models suggest that the hippocampus is well situated to maintain a neural representation of the CS during the trace interval (Wallenstein *et al.*, 1998). Collectively, these findings support a role for the dorsal hippocampus in maintenance of the CS during the trace interval.

Enhancement of trace fear conditioning by dorsal hippocampal nicotine infusion as well as deficits induced by infusion of high-affinity nicotinic receptor antagonist DHβE suggest that nAChRs are involved in maintenance of a CS representation in the dorsal hippocampus during the trace interval. However, there is a mismatch between

effects of systemic DHβE and dorsal hippocampal DHβE. Systemic administration of DHβE has no effect on trace conditioning (Raybuck & Gould, 2009), whereas the present studies show that dorsal hippocampal DHβE infusion produces deficits in trace conditioning. This may be because in the dorsal hippocampus DHβE produces deficits in trace conditioning, whereas in the medial prefrontal cortex DHβE enhances trace conditioning. Thus, systemic DHβE administration may produce deficits in dorsal hippocampal support for trace fear conditioning, which could be obscured by an enhancement of medial prefrontal cortical support for trace fear conditioning. This would suggest that the medial prefrontal cortex can compensate for deficits in dorsal hippocampal function in this task, although, further investigation is necessary to confirm this relationship. However, it is also possible that studies using systemic administration have used the wrong doses, or that DHβE does not adequately cross the blood brain barrier following systemic administration. Collectively, these findings suggest a role for nAChRs in the dorsal hippocampus in trace fear conditioning.

One mechanism through which nicotinic acetylcholinergic signaling in the dorsal hippocampus could support trace fear conditioning is long-term potentiation (LTP) (Hasselmo, 2006). Nicotine has been shown to enhance induction of some forms of LTP, a model of cellular and molecular processes thought to underlie learning and memory (Fujii *et al.,* 1999; He *et al.*, 2000; Matsuyama *et al.*, 2000; Nakauchi *et al.*, 2007; Sawada *et al.*, 1994; Wang *et al.*, 2001; Welsby *et al.*, 2006; Welsby *et al.*, 2009; Yamazaki *et al.*, 2002; Yamazaki *et al.*, 2005; Yamazaki *et al.*, 2006). Additionally, computational models suggest that LTP between DG and CA3 can support maintenance of a CS across the trace interval (Levy *et al.,* 2005; Rodriguez & Levy, 2001). This is

supported by cellular recordings from the dorsal hippocampus showing that firing in the DG increases during the trace interval (Gilmartin & McEchron, 2005). Further, trace fear conditioning is shown to be associated with the strength of LTP induction in the dorsal hippocampus (Song *et al.*, 2008). Thus, it seems likely that nicotine's effects on trace fear conditioning could be mediated by the enhancement of LTP-like processes; although, further investigation will be necessary to confirm this link.

Role of the ventral hippocampus in trace fear conditioning

The ventral hippocampus is critically involved in both acquisition and retrieval of fear conditioning. Lesions to the ventral hippocampus produce deficits in fear conditioning (Bast *et al.,* 2001; Esclassan *et al.*, 2009; Maren & Holt, 2004; Rudy & Matus-Amat, 2005; Yoon & Otto, 2007; Zhang *et al.*, 2001). This may be because the ventral hippocampus serves as a conduit for information between the medial prefrontal cortex, dorsal hippocampus and amygdala (Hobin *et al.,* 2006; Maren & Fanselow, 1995; Morgane *et al.*, 2005). In support of this, the ventral hippocampus is interconnected with the dorsal hippocampus and medial prefrontal cortex (Morgane *et al.*, 2005), which are both critically involved in trace fear conditioning (McEchron *et al.,* 1998; Quinn *et al.*, 2008; Runyan *et al.*, 2004); and the amygdala (Pitkänen *et al.*, 2000), which is critically involved in both acquisition and expression of fear conditioning (Fanselow & LeDoux, 1999; Paré *et al.*, 2004). Additionally, some neurons in the ventral hippocampus extend projections to both the amygdala and medial prefrontal cortex (Ishikawa & Nakamura, 2006). It has also been proposed that lesions to the ventral hippocampus may alter amygdala function, resulting in deficits in fear learning, expression, and anxiety in

general (Bannerman *et al.,* 2003 ; Bannerman *et al.*, 2004; Degroot & Treit, 2004; Kjelstrup *et al.*, 2002; Maren & Holt, 2004; Pentkowski *et al.*, 2006). Thus, the connectivity of the ventral hippocampus may allow it to regulate fear expression.

In support of the ventral hippocampus regulating fear expression, studies suggest that the ventral hippocampus is involved in extinction. Extinction is not an unlearning of old associations, but rather a process in which new associations interfere with expression of old associations. Ventral hippocampal lesions produce deficits in behavioral suppression associated with extinction (Clark *et al.*, 1992), and inactivation of the ventral hippocampus produces deficits in context specific extinction of delay-cued fear conditioning (Hobin *et al.*, 2006). Further, at the cellular level, extinction training is followed by increases in synaptic efficacy between the ventral hippocampus and the medial prefrontal cortex, and these changes precede alteration of medial prefrontal cortical-thalamic connectivity, suggesting that the ventral hippocampus could be critically involved in acquisition of extinction (Hugues & Garcia, 2007). In addition, extinction of appetitive conditioning critically depends on cholinergic projections to the ventral hippocampus from nuclei in the medial septum (Blaker *et al.*, 1984). Thus, the effects of nicotine in the ventral hippocampus at testing could relate to activation of extinction-related processes suppressing expression of the learned response. However, this mechanism cannot explain the effects of nicotine infusion into the ventral hippocampus at training. Nicotine infusion into the ventral hippocampus may disrupt information flow from the dorsal hippocampus and ventral hippocampus to the amygdala resulting in a learning deficit at training. Therefore, if nicotine in the ventral

hippocampus disrupts the integration of information across brain regions involved in trace and contextual fear conditioning this could disrupt both learning and recall.

Role of the medial prefrontal cortex in trace fear conditioning

Trace fear conditioning depends upon the medial prefrontal cortex, and evidence suggests that this area may be a permanent site of memory storage for trace conditioning (Quinn *et al.,* 2008; Runyan *et al.*, 2004), but, the exact role of the medial prefrontal cortex in trace conditioning is not understood. A working memory dependent model of trace fear conditioning would predict that the dorsal hippocampus and medial prefrontal cortex are involved in maintenance of the CS in working memory during the trace interval (Carter *et al.,* 2003; Gilmartin & McEchron, 2005). However, these two regions may play more specific roles. While, the dorsal hippocampus could maintain a representation of the CS during the trace interval (Levy *et al.,* 2005; Rodriguez & Levy, 2001; Wallenstein *et al.*, 1998), the medial prefrontal cortex may be involved in assigning salience and predictive value to stimulus representations maintained by the dorsal hippocampus (Bishop, 2008; Ventura *et al.*, 2007). Thus, during trace fear conditioning the medial prefrontal cortex would determine which of the stimuli present during the training session best predicted US occurrence. In support of this role, the medial prefrontal cortex receives afferent projections from the dorsal hippocampus as well as thalamic regions responsible for processing visceral stimuli (Heidbreder & Groenewegen, 2003; Hoover & Vertes, 2007; Vertes, 2004), and single neurons in the prelimbic region of the medial prefrontal cortex fire in response to the CS and US during trace conditioning (Gilmartin & McEchron, 2005). Thus, the medial prefrontal cortex could

form associations between conditional stimulus representations maintained by the dorsal hippocampus, and visceral stimuli.

The present findings suggest that the role of nAChRs in the medial prefrontal cortex is to regulate learning related plasticity. In the medial prefrontal cortex nicotine infusion can enhance trace conditioning, as can either high- or low-affinity nicotinic antagonists, suggesting that nicotine acts in this brain region by desensitizing receptors. Indeed, desensitization of nAChRs is thought to decrease tonic dopamine release, increasing the salience of phasic dopamine release (Picciotto *et al.,* 2008; Zhang *et al.*, 2009), which is thought to signal significant stimuli and induce learning related plasticity (Grace, 1993). Indeed, activation of dopaminergic D1 receptors is a critical event for trace fear conditioning (Runyan & Dash, 2004), and, multiple studies have shown that nicotine infusion into the medial prefrontal cortex can enhance dopamine release (Cao *et al.,* 2005; Rao *et al.*, 2003; Rodvelt *et al.*, 2008; Rossi *et al.*, 2005; Shearman *et al.*, 2005; Singer *et al.*, 2004), although these studies do not differentiate between tonic and phasic dopamine release. Thus, it may be that in the medial prefrontal cortex nicotine decreases tonic dopamine levels but increases phasic dopamine release to salient stimuli, explaining enhancement of trace fear conditioning by medial prefrontal nicotine administration. However, further investigation will be necessary to determine if the actions of medial prefrontal nicotine on trace fear conditioning are mediated by increases in dopaminergic activity.

Figure 30.

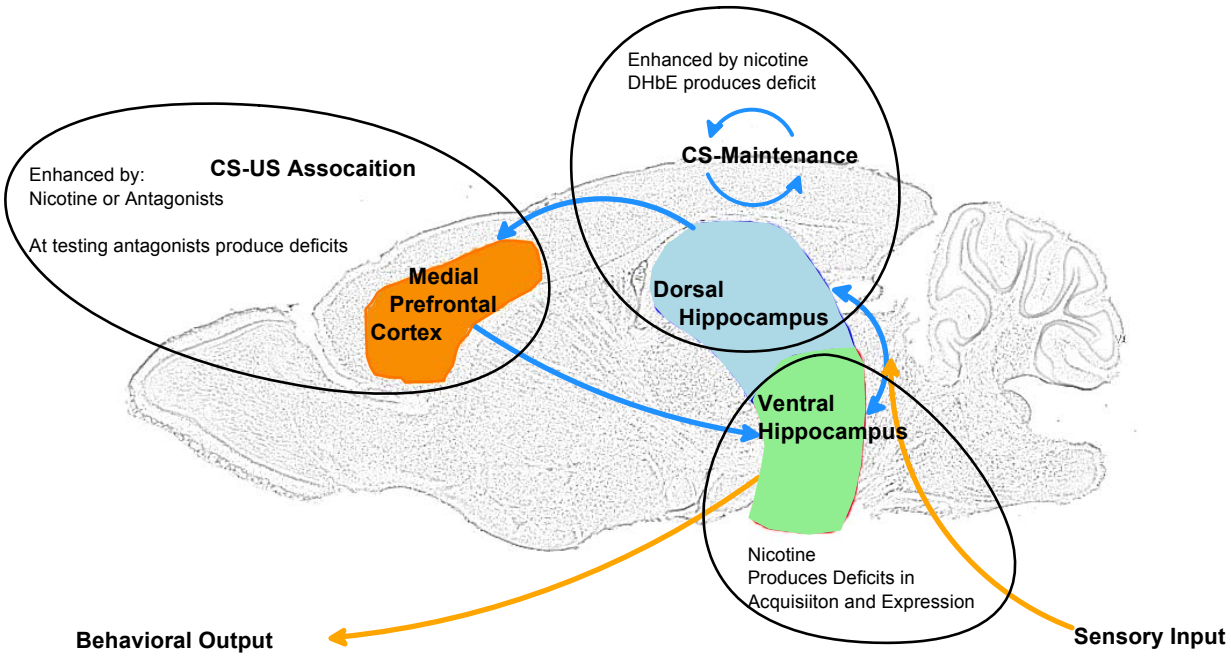

Figure 30: A model of the substrates of trace fear conditioning, and the role of nAChRs. High-affinity nAChRs in the dorsal hippocampus facilitate CS maintenance during the trace interval. Desensitization of nAChRs in the medial prefrontal cortex increases salience of the CS and facilitates association of the CS and US. Activation of nAChRs in the ventral hippocampus can suppress both learning and expression of trace and contextual conditioning.

Implications of the effects of local nicotine and nicotinic antagonist infusion for theories of trace fear conditioning

In demonstrating that nicotine and nicotinic antagonists in the dorsal hippocampus have different effects on trace and contextual fear conditioning, the present studies reinforce the idea that trace conditioning and contextual conditioning are supported by different dorsal hippocampal mechanisms, a conclusion with direct implications for theories of trace conditioning. Contextual bridging explains how the CS could cross the trace interval as well as why the hippocampus is necessary for learning (Marchand *et al.*, 2004). Contextual conditioning, which is dependent on the dorsal hippocampus, is

thought to be the sum of a multi-modal stimuli present in a particular context, through feature binding these stimuli are associated with each other and come to form a coherent representation of a context (Fanselow, 2000; Rudy *et al.*, 2004). Contextual bridging assumes that the dorsal hippocampus is involved in trace fear conditioning because there is a critical contextual component of this task, thus a common mechanism in the dorsal hippocampus supports both trace and contextual conditioning (Marchand *et al.*, 2004). However, the present studies demonstrate that in a 2-pairing trace fear conditioning paradigm different doses of nicotine enhance trace or contextual conditioning in the same animals. Specifically, infusion of nicotine at 0.35 μg/side enhanced contextual fear conditioning but did not affect trace conditioning; and infusion of nicotine at 0.09 μg/side enhanced trace conditioning, but did not affect contextual conditioning. Additionally, the present findings show that in a 5-pairing trace conditioning paradigm, infusion of the high-affinity nicotinic antagonist DHβE into the dorsal hippocampus produces deficits in trace but not contextual conditioning. These findings demonstrate that the roles of nAChRs within the dorsal hippocampus in trace and contextual fear conditioning are dissociable, suggesting that they occur through different mechanisms. Other reports also suggest that trace and contextual conditioning are supported by different dorsal hippocampal mechanisms. Infusion of NMDA receptors antagonist APV at testing produces deficits in trace but not contextual conditioning, suggesting that NMDA receptor dependent processes in the dorsal hippocampus are involved in expression of trace conditioning but not contextual conditioning (Quinn *et al.*, 2005). Additionally, immediate early gene expression patterns in the dorsal hippocampus are different between trace and delay fear conditioning, with increased expression in the CA3

subregion in trace conditioned as compared to delay conditioned animals, suggesting that trace conditioning activates a different population of hippocampal neurons than delay conditioning (Weitemier & Ryabinin, 2004). Collectively, these findings suggest that different mechanisms in the dorsal hippocampus support trace and contextual fear conditioning. This directly contradicts the predictions of contextual bridging.

An alternate theory to contextual bridging is that working memory supports trace conditioning. A working memory dependent model of trace fear conditioning explains hippocampal involvement in this task as well as the involvement of the medial prefrontal cortex. Further this model explains findings that attentional distracters produce deficits in trace conditioning, that awareness of the CS-US relationship is a strong predictor of strength of conditioning, and why concurrent n-back task performance produces deficits in trace conditioning (Carter *et al.,* 2003; Han *et al.*, 2003; Knight *et al.*, 2006; Weike *et al.*, 2007). Further, both working memory and trace fear conditioning depend upon dopaminergic transmission in the prefrontal cortex (Arnsten, 1997; Dash *et al.*, 2007; Romanides *et al.*, 1999; Runyan & Dash, 2004). The present findings suggest involvement of nicotinic signaling in both dorsal hippocampus and medial prefrontal cortex in trace fear conditioning. This is consistent with a role for cholinergic modulation of working memory and with the well studied role of these brain regions in a number of working memory dependent tasks (Dash *et al.,* 2007; Dawkins *et al.*, 2007; Granon *et al.*, 1994; Granon *et al.*, 1995; Granon *et al.*, 2003; McGehee, 2007; Raybuck & Gould, 2009; Swan & Lessov-Schlaggar, 2007). Collectively these findings support a working memory-dependent model of trace fear conditioning, and suggest that

cholinergic signaling may play a critical role in processes that support acquisition of trace conditioning.

Future Directions

The present findings demonstrate differential effects of nicotine on trace fear conditioning and critical and modulatory roles for nAChRs in the dorsal hippocampus, ventral hippocampus, and medal prefrontal cortex, but in doing so these studies also pose a number of questions for future investigation. Previous research shows that acute nicotine enhances trace fear conditioning, whereas withdrawal from chronic nicotine produces deficits in trace fear conditioning (Raybuck & Gould, 2009). While the effects of nicotine withdrawal on contextual fear conditioning have been localized to the dorsal hippocampus (Davis & Gould, 2009), the present findings demonstrate that the dorsal hippocampus and medial prefrontal cortex are sites in which acute nicotine could act to enhance trace fear conditioning and that acetylcholinergic receptors play an important role in trace fear conditioning. Thus, it is possible that either or both of these regions may be critical to nicotine's chronic and withdrawal effects on trace conditioning. Additionally, it is possible that the medial prefrontal cortex also plays a role in the chronic and withdrawal effects of nicotine on contextual fear conditioning. Future research should investigate whether local infusion of nicotinic antagonists into these brain regions is able to precipitate withdrawal deficits in these tasks in chronic nicotine treated animals, and if nicotine withdrawal induced deficits in trace and contextual conditioning can be ameliorated by infusion of drugs into these regions.

The present studies show clear effects of nicotine and nicotinic antagonists in the medial prefrontal cortex on trace fear conditioning. While these effects may be best explained by modulation of dopaminergic signaling, the potential link between modulation of dopamine release in the medial prefrontal cortex by nicotine and cognition has not yet been investigated. Future studies should use pharmacological manipulations to determine if activation of dopaminergic receptors in the medial prefrontal cortex is critical to the enhancing effects of nicotinic agents on trace fear conditioning.

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