Acetylcholine levels in the prefrontal cortex and hippocampus during trace and delay conditioning

Mary Melissa Flesher
ACETYLCHOLINE LEVELS IN THE PREFRONTAL CORTEX AND HIPPOCAMPUS DURING TRACE AND DELAY CONDITIONING

A Thesis
Presented to the
Faculty of
California State University,
San Bernardino

In Partial Fulfillment
of the Requirements for the Degree
Master of Arts
in
Psychology:
General-Experimental

by
Mary Melissa Flesher
December 2008
ACETYLCHOLINE LEVELS IN THE PREFRONTAL CORTEX AND HIPPOCAMPUS DURING TRACE AND DELAY CONDITIONING

A Thesis
Presented to the
Faculty of
California State University,
San Bernardino

by
Mary Melissa Flesher
December 2008

Approved by:

Dr. Allen E. Butt, Chair, Psychology

Dr. Cynthia A. Crawford

Dr. Yuchin Chien

12 - 1 - 08
Date
ABSTRACT

Damage to the medial prefrontal cortex (mPFC) or hippocampus (HPC) causes a dissociation of impairment in trace and delay conditioning. Unlike delay conditioning, trace conditioning requires cognitive processes including attention and declarative memory, which depend on the mPFC and HPC, respectively. The cholinergic basal forebrain system modulates activity in widespread regions including mPFC and HPC, suggesting that acetylcholine (ACH) may also make different contributions to trace and delay conditioning. The goal of the current experiment was to examine the pattern of ACh release in mPFC and HPC during performance in trace and delay appetitive conditioning. Microdialysis probes were implanted in the mPFC and HPC of rats pretrained in both the delay and trace conditioning paradigms. Dialysate samples were collected during a quiet baseline period and during subsequent trace and delay conditioning performance. ACh was quantified using high performance liquid chromatography with electrochemical detection techniques. As hypothesized, it was found that ACh levels in the mPFC were greater during performance of trace conditioning than during performance of delay conditioning. ACh levels in the HPC were also found to be greater during performance of trace conditioning than
during performance of delay conditioning, although HPC ACh levels were lower than mPFC levels during trace conditioning. Testing-induced ACh release during trace conditioning exceeded baseline levels in both brain regions, whereas testing-induced ACh levels during delay conditioning were not significantly greater than baseline levels. Collectively, findings from this experiment demonstrate a continued involvement of cholinergic modulation in mPFC during performance of a previously acquired trace conditioning task, where cholinergic activity in the mPFC exceeds that observed in the HPC during trace conditioning and exceeds the level of cholinergic activity observed in either the mPFC or HPC during delay conditioning.
ACKNOWLEDGMENTS

First and foremost, I would like to thank my mentor and thesis chair Dr. Allen E. Butt for his guidance and never-ending support throughout this project. The invaluable training I have received from him has not only provided me the tools and advice to be successful in this project and in my graduate education at CSUSB, but it has also allowed me the opportunity to grow as a scientist and be better prepared for future challenges. He has truly gone above and beyond what was required and this has no doubt allowed me a myriad of opportunities and successes that I would never have been offered without his help.

I also owe thanks to my thesis committee members Dr. Yuchin Chien and Dr. Cynthia Crawford for their assistance in editing and providing recommendations for this project. Their patience and expertise throughout the course of this work are greatly appreciated.

I would like to thank the students in Dr. Butt’s laboratory for their assistance in the execution of this project. I additionally want to thank my family and friends for their continual encouragement and understanding.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>iii</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>viii</td>
</tr>
<tr>
<td><strong>CHAPTER ONE: PAVLOVIAN TRACE AND DELAY CONDITIONING IN HUMANS</strong></td>
<td></td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>The Role of Awareness in Human Pavlovian Conditioning</td>
<td>5</td>
</tr>
<tr>
<td><strong>CHAPTER TWO: THE ROLE OF THE HIPPOCAMPUS IN TRACE AND DELAY PAVLOVIAN CONDITIONING</strong></td>
<td></td>
</tr>
<tr>
<td>Introduction</td>
<td>14</td>
</tr>
<tr>
<td>Lesion Studies</td>
<td>15</td>
</tr>
<tr>
<td>Pharmacological Studies</td>
<td>29</td>
</tr>
<tr>
<td>Microdialysis Studies</td>
<td>33</td>
</tr>
<tr>
<td>Genetic Knockout Studies</td>
<td>35</td>
</tr>
<tr>
<td>Developmental Studies</td>
<td>39</td>
</tr>
<tr>
<td><strong>CHAPTER THREE: THE ROLE OF THE PREFRONTAL CORTEX IN TRACE AND DELAY PAVLOVIAN CONDITIONING</strong></td>
<td></td>
</tr>
<tr>
<td>Introduction</td>
<td>46</td>
</tr>
<tr>
<td><strong>CHAPTER FOUR: THESIS PROPOSAL</strong></td>
<td></td>
</tr>
<tr>
<td>Introduction</td>
<td>53</td>
</tr>
<tr>
<td><strong>CHAPTER FIVE: THESIS EXPERIMENT</strong></td>
<td></td>
</tr>
<tr>
<td>Methods</td>
<td>59</td>
</tr>
<tr>
<td>Guidelines for Animal Use</td>
<td>59</td>
</tr>
<tr>
<td>Animals</td>
<td>59</td>
</tr>
</tbody>
</table>

vi
Apparatus ........................................ 59
Surgery ........................................... 61
General Procedure ............................... 61
Statistical Analyses ............................. 65
Histology ......................................... 66

CHAPTER SIX: RESULTS AND DISCUSSION

Results ........................................... 67
Probe Placement Verification ................. 67
Behavioral Results ............................. 67
Neurochemistry Results ....................... 72
Discussion ....................................... 79

REFERENCES .................................... 86
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Probe Placement</td>
<td>68</td>
</tr>
<tr>
<td>2</td>
<td>Conditioned Responding Acquisition</td>
<td>70</td>
</tr>
<tr>
<td>3</td>
<td>Adaptive and Non-adaptive Responding</td>
<td>73</td>
</tr>
<tr>
<td>4</td>
<td>Acetylcholine Efflux</td>
<td>75</td>
</tr>
<tr>
<td>5</td>
<td>Acetylcholine Efflux in Non-learners</td>
<td>78</td>
</tr>
</tbody>
</table>
CHAPTER ONE

PAVLOVIAN TRACE AND DELAY CONDITIONING IN HUMANS

Introduction

Pavlovian conditioning is a form of associative learning in which an originally neutral conditioned stimulus (CS) becomes associated, through pairing, with a biologically meaningful unconditioned stimulus (US). This procedure can be carried out using a variety of different temporal CS-US arrangements. The fastest learning typically comes from arranging the CS so it begins prior to and then coterminates with the onset of the US. This arrangement of stimuli is termed delay conditioning and is a form on non-declarative memory. In trace conditioning, which is more difficult to learn, the CS offset precedes the US onset by a fixed interval of time. Unlike delay conditioning, trace conditioning requires cognitive processes including attention and declarative memory, which depend on the prefrontal cortex and hippocampus (HPC), respectively (see Kesner, 2005). In a related procedure, termed long-delay conditioning, the CS offset co-terminates with the US onset similar to the arrangement in delay conditioning, however the CS occurs for a longer period of time, typically appreciating the inter-stimulus
interval (ISI) used in trace conditioning. This task is used to test for differences in performance as a function of increased ISI. Because the trace conditioning paradigm incorporates both increased ISI as well as a working memory-dependent time gap, the long-delay conditioning paradigm provides a useful control to determine which of these two components is responsible for deficits in trace conditioning. The present review will focus on differentiating the role of awareness in trace and delay conditioning.

Trace, long-delay, and delay conditioning are vital tools for discovering the role of specific brain regions in learning and memory in non-human animals. The major limitation in this area of study is that it is very difficult to operationalize concepts like awareness and declarative memory in animal models where only observable behavior is measurable. Having a human research equivalent to these animal studies allows greater generalization to the animal research and therefore provides a more viable means of measuring difficult concepts through the use of lesioning, microdialysis, and genetic manipulations.

Woodruff-Pak and Jaeger (1998) attempted to find predictors of age differences in eyeblink conditioning. They hypothesized that blink reaction time as well as
timed-interval tapping would predict performance on eyeblink conditioning, with detriments in one task predicting detriments in the other. They found that age accounted for the majority of the variance in acquisition in the task, with increased age predicting decreased performance on the task. They also found that the cerebellar dependent timed-interval tapping task performance positively correlated with acquisition of the conditioning task. They concluded that age-related deficits in cerebellar function negatively affect timing and associative learning.

Finkbiner and Woodruff-Pak (1991) tested three age groups in trace conditioning with an extended ISI to assess age related differences in this task. They found no difference between the young (17-22 yrs old) and middle-aged groups (39-52 yrs old); however the old group (64-81 yrs old) had poorer performance than both other groups. The age difference found here was manifest in the asymptotic level each group reached and not in the overall learning curve. The deficit, which occurred only in the older age group with the extended ISI, contradicted previous literature with a shorter ISI showing that trace conditioning begins to show age detriments around middle-age. Finkbiner and Woodruff-Pak (1991) believe that this
may be due to the extended ISI giving the middle-aged group more time to respond.

Herbert, Stanton, and Eckerman (2003) tested differences in adults and infants using standard puff-tone eyeblink delay, long delay, and trace conditioning. They found that both adults and infants were able to learn and reach asymptote in the delay conditioning task. While adults learned more rapidly in the beginning of training both groups were at comparable asymptote by the second trial. Infants were found to have deficits in both the long-delay and trace conditioning tasks as compared to adults who exhibited robust learning in both tasks. They believe that the deficits found in infants' conditioned response (CR) acquisition during long-delay and trace conditioning may be due to the increased ISI (present in both tasks) and not the working memory component of the trace conditioning (only present in the trace task). This may be due to the prolonged postnatal development in the cerebellar structures, specifically the cerebellar-brainstem learning circuit, which is not yet fully developed in 5 mo. old infants.

Taken together, the human research on delay, long-delay, and trace conditioning show a clear discernment across age groups. It can be seen that the neural
circuitry for delay conditioning develops early in life and maintains function throughout the normal life span. Long-delay conditioning circuitry however does not develop until at least after 5 mo. of age. Trace conditioning neural circuitry (in regions such as the HPC and frontal cortex) develop at a relatively slow rate and begin to deteriorate with advanced age (correlating with the natural loss of hippocampal cells occurring late in life). This demonstrates a useful tool in utilizing trace conditioning acquisition to assess proper hippocampal function.

The Role of Awareness in Human Pavlovian Conditioning

A great deal of research has been conducted assessing the role of conscious awareness (i.e. a cognitive reaction to internal or external stimuli) in Pavlovian trace and delay conditioning. Despite a plethora of research available the role of awareness in delay conditioning remains largely under debate (for review see Lovibond & Shanks, 2002), while there is a much greater consensus for the vital role of awareness in trace conditioning. Research from the 1970’s and early 1980’s found that awareness of the relationship between the CS and US was correlated with successful acquisition of delay eyeblink
conditioning (Benish & Grant, 1980; Ross & Nelson, 1973). However, more recent research has come to conclusions inconsistent with these findings demonstrating that awareness of CS-US contingencies is unnecessary for delay conditioning (Clark & Squire, 1998; Smith, Clark, Manns, & Squire, 2005).

Clark and Squire (1998) tested the role of awareness in normal adults and temporal-lobe amnesic patients in both trace and delay conditioning. Participants were exposed to either a trace or delay differential conditioning procedure in which a tone or white noise CS+ was paired with an air puff (US) to the eye, while the counterbalanced CS- was presented alone. For the delay conditioning protocol a 700 ms or 1250 ms CS was used. For trace conditioning a 250 ms or 500 ms trace interval (TI) was utilized. As can be seen the delay conditioning task was technically a long-delay task, although not mentioned in the article. In order to equate performance the ISIs for the amnesic patients were first given the shorter interval and moved up to the longer interval CS or TIs (at which time they were assessed for performance), while normal patients were randomly assigned to a specific length. While participants underwent these procedures they were instructed to watch a silent movie. After completion
of the movie participants were given a questionnaire asking questions about the relationship between the CS and US and filler questions about the movie.

They found that amnesic patients were able to acquire the delay, but not trace, conditioning task. Their questionnaire scores demonstrate that they were unaware of the CS-US contingency for both tasks. For normal patients, awareness of CS-US contingency was required for successful acquisition of trace conditioning, but was not necessary for the delay conditioning task. In the delay conditioning procedures normal participants had equal performance regardless of their awareness of the CS-US contingency, while for trace conditioning only those that were aware of the contingency were able to acquire the task.

Clark and Squire (1998) argue that in order to acquire the trace conditioning task the participant must be aware of and remember information about the structure of the task, because the separation of the CS and US make the task unable to be processed in an automatic manner. The authors suggest that this degree of complexity requires the additional recruitment of the neocortex and HPC, not necessary for the similar delay conditioning task. These results demonstrate that trace conditioning is a viable task for measuring declarative memory. This is
consistent with previous studies showing that hippocampal damage (which is damaged in the amnesic patients in this study) causes a selective impairment in trace, but not delay conditioning (Kim, Clark, & Thompson, 1995; Quinn et al., 2002).

In an article that further examined trace conditioning, Manns, Clark, and Squire (2000) assessed the temporal relationship between the moment of becoming aware of contingencies in a task and the time of trace eyeblink conditioning acquisition in older adult humans. They hypothesized that participants should be able to learn the task so long as they are aware of the contingency.

They had 26 older adults (age range: 47-79) with similar education levels undergo trace eyeblink conditioning in one of two groups. One group was asked to predict the airpuff by pressing a button when they felt an airpuff was about to occur. The other group was asked to predict when they were going to blink their eyes by pressing a button before a predicted eyeblink. Participants were told that the experiment they were partaking in was examining how distraction influences learning and memory. They were then instructed to watch a silent movie and try to remember as much of it as they could. After the task they were required to fill out a 17
question survey measuring their knowledge of the silent movie as well as their awareness of the CS-US contingency.

As hypothesized, they found that participants were able to learn the trace conditioning task only if they became aware of the contingency present between the CS and US. Participants who were asked to predict the airpuff acquired the trace conditioning task, as well as the ability to accurately predict the CS, much more successfully than the group asked to predict their eyeblinks, which acquired neither the task nor knowledge of the CS-US contingency. These results demonstrate that awareness of the relationship between the CS and the US in trace eyeblink conditioning facilitates acquisition of the task. They conclude that acquisition and awareness of the conditioning contingencies occur in parallel during training in trace conditioning.

Knuttinen, Power, Preston, and Disterhoft (2001) also examined Pavlovian trace and delay discrimination eyeblink conditioning in young (25-35 yrs old) and old (65-75 yrs old) humans to assess the role of age and awareness on the acquisition of these tasks. They had participants watch a silent movie while being presented with the trace and delay conditioning tasks. Participants were surveyed afterwards to assess their awareness of the movie as well
as the contingency between the CS and US. For delay conditioning an 850-ms or 1350-ms tone or white noise was used as either the CS+ (predictive of US) or CS- (predictive of no CS), with an eyepuff US. For trace conditioning the same parameters were used with only an addition of a 500-ms or 1,000-ms TI following the offset of the CS+.

They found that awareness of stimulus contingencies was positively correlated with successful acquisition of the delay and trace paradigms they used, regardless of age. They also found age dependent differences in the trace, but not delay conditioning task, with the most severe deficit due to age occurring during the task with the trace conditioning task with the longest TI. More young adults were found to be aware of the contingency than older adults. They believe that additional attentional demands (most likely cerebellum dependent) are placed on multi-cued tasks as opposed to single-cue tasks. They argue that differential neural systems may participate in a parallel fashion during declarative and behavioral aspects of learning. They also conclude that older adults may be less adept at multitasking, requiring that they devote more attention to each individual task.
These studies demonstrate that delay conditioning is not as reliant on awareness as previously believed. They show that awareness is a vital component of trace, but not delay, eyeblink conditioning in humans. This gives support to the idea that delay conditioning is a form of non-declarative memory, while trace conditioning is a form of declarative memory (see Clark, Manns, & Squire, 2001). This dissociation of cognitive requirements in these tasks is particularly interesting because the stimuli presented, as well as the CR elicited, from each of these tasks are identical, with the only difference being temporal contiguity of the CS and US. Yet, despite their functional similarities they appear to be utilizing distinct and separable memory systems. With the ever-growing field of research on multiple memory systems this makes trace and delay conditioning an attractive option to study declarative and non-declarative memory.

The separable nature of the two tasks was especially evident in Clark and Squire’s (1998) finding that patients with temporal-lobe amnesia were able to readily acquire delay conditioning, even though they were unable to learn trace conditioning. The fact that their performance was similar to the unaware participants in this study supports the idea that the HPC and related structures are at least
partially responsible for awareness in the trace conditioning task (see Thompson & Kim, 1996). Knuttinin and colleagues (2001) finding that trace, but not delay, conditioning acquisition is retarded in older people, as compared to a younger group, shows that the cognitive and neural processes responsible for these two types of learning undergo differential aging related damage. These distinctions are particularly relevant because they demonstrate that distinctions between trace and delay conditioning have practical implications outside of laboratory, including utilizing them to assess proper hippocampal functioning and natural age related cognitive deficits.

Trace and delay conditioning are vital tools for discovering the role of specific brain regions in learning and memory in non-human animals. The major limitation in this area of study is that it is very difficult to operationalize concepts like awareness, attention, and declarative memory in animal models where only observable behavior is measurable. Having a human research equivalent to these animal studies allows greater generalization to the animal research and therefore provides a more viable means of measuring difficult concepts through the use of lesioning, microdialysis, and genetic manipulations.
Awareness is difficult to assess in non-human animals and yet with the wealth of knowledge demonstrating it is a vital aspect for proper acquisition of the trace conditioning task in humans it can be implied that similar function is occurring during animal models of trace conditioning. The research demonstrates that Pavlovian trace conditioning is a valuable paradigm for studying declarative memory, because of both the hippocampal dependent nature of it as well as the awareness-demanding features of this task.
CHAPTER TWO
THE ROLE OF THE HIPPOCAMPUS IN TRACE AND DELAY PAVLOVIAN CONDITIONING

Introduction

In recent years the role of the HPC in trace and delay conditioning has become of particular interest to researchers because of its presumed role in memory consolidation, contextual conditioning, declarative memory, and timing, although its exact role in and the extent to which it participates in each of these is still under much scrutiny. The specific function of each of these aspects of hippocampal function in trace and delay Pavlovian conditioning will be discussed in detail below.

The role of the HPC in Pavlovian conditioning is multifaceted as it is comprised of multiple distinct regions, including the CA1, CA2, CA3, dentate gyrus, and perirhinal cortex, all of which may participate differently in Pavlovian conditioning. This chapter will discuss many of the roles of the HPC during trace and delay conditioning. A variety of different methods, including full and partial hippocampal lesions, pharmacological manipulations, microdialysis studies,
genetic knockout of specific hippocampal components, and developmental studies are discussed.

Lesion Studies

It has been previously reported that basal forebrain cholinergic lesions cause a selective impairment in trace conditioning but not delay conditioning (Butt et al., 2007). These findings are consistent with reports showing that damage to the PFC or HPC, both of which receive input from the cholinergic basal forebrain, similarly causes a dissociation of impairment in trace and delay conditioning.

It has been demonstrated that the HPC is necessary for consolidation of trace, but not delay, conditioning as is evident with hippocampal lesions one day after acquisition of either task (Kim et al., 1995; Quinn, Oommen, Morrison, & Fanselow, 2002). However, the HPC has been demonstrated to not be necessary for post consolidation performance of trace, or delay, conditioning. Specifically it was found that hippocampal lesions one month after acquisition of trace conditioning had no detrimental effect on performance on the trace conditioning task, although lesions one day after
acquisition caused a selective impairment of trace, but not delay, conditioning (Kim et al., 1995).

Fendt, Fanselow, and Koch (2005) examined the specific role of the dorsal HPC (dHPC) in trace fear conditioning by testing the effects excitotoxic lesions of the dHPC have on a fear-potentiated acoustic startle response during trace and context fear conditioning. Animals in this experiment received either bilateral dHPC lesions by N-methyl-D-aspartate (NMDA) injection or sham, vehicle-injected, surgery. After recovery, animals underwent an assessment day in which they were placed in a startle chamber and, after a 5 min baseline period, were presented with 8 startling white noise USs pseudorandomly presented half the time alone and the other half 10 s after a tone CS. This was done to assess the acoustic startle response in naïve rats during the tone CS. On the following 4 days of testing, animals were again placed in the startle chambers and habituated for 5 min after which they underwent 5 trace fear conditioning pairings with a tone CS being followed 10 s later by a footshock US. After a 100 s wait period they then received 5 white noise startle stimuli for habituation. They then underwent the same procedure as the assessment day with 8 startling white noise stimuli pseudorandomly presented half the time.
alone and the other half 10 s after a tone CS. The baseline measurements were used to assess context conditioning and the mean differences between the startling white noise US alone and startling white noise US paired with tone CS were used to measure trace fear conditioning.

It was found that the dHPC lesions did not affect contextual conditioning. However, dHPC lesions did prevent acquisition of the trace fear conditioning. The authors argue that this difference may be the result of other regions of the cortex compensating for dHPC damage in contextual conditioning. They believe that these regions may be inhibited during times of normal HPC function. This is the basis of the explanation of why pretraining HPC lesions have the most impact on the acquisition of tasks that have processing demands exceeding that which the other cortical areas are able to compensate for, such as trace conditioning.

To expand on these findings Rogers, Hunsaker, and Kesner (2006) tested the role of various parts of the HPC to discern their implications in trace fear conditioning. They focused on the CA1 region because of its involvement in temporal processing. They hypothesized that lesions of the dorsal CA1 would result in retardation of contextual
conditioning and inhibit conditioned responding during the TI. Lesions of the ventral CA1 were hypothesized to attenuate conditioned responding during the TI and CS and to possibly increase overall activity levels.

Animals were randomly assigned to one of four surgery groups; an ibotenic acid lesion of dorsal or ventral CA1, or a vehicle control dorsal or ventral CA1 sham group. They were tested in a fear-conditioning chamber placed in a room with various contextual cues. This chamber was also used for acquisition and contextual retention tests. A contextually different second chamber located in a separate room with different visual cues was used for the testing phase of this experiment.

Day one consisted of an acquisition phase in which animals were placed in the fear-conditioning chamber and given a 2 min stimulus free baseline followed by 15 tone-trace-shock pairing trials. These pairings consisted of the presentation of a 32 s tone CS followed by a 10 s TI after which a 2 s electric foot shock US was given. Analysis of conditioned freezing responses, which were categorized as an animal's lack of movement aside from respiration, was done from a video recording of the session. On day two, animals were tested for contextual conditioning in the fear-conditioning chamber used during
acquisition. Testing for contextual conditioning consisted of leaving the animal in the chamber for 8 min in the absence of stimuli. On the final day of testing, animals were placed in the contextually different Plexiglas chamber to test retention of the CR. They were given a 2 min stimulus-free baseline and then presented 15 tone-trace trials similar to those provided on the acquisition day minus the shock US.

It was found that during acquisition all groups performed at the same level. In the contextual retention test it was found rats in the ventral CA1 lesion group showed significantly less freezing than the dorsal CA1 lesion group or the sham lesion groups. Those with dorsal CA1 lesions were also found to have significantly fewer freezing responses than the sham lesioned groups. In the trace retention test rats with ventral CA1 lesions showed significantly less conditioned responding as compared to the dorsal CA1 lesion and sham lesion groups. There was no difference in performance between the dorsal CA1 lesion group and the sham lesion groups. No differences in CR to the tone were found, with all groups showing conditioning to the tone.

Contrary to the authors' predictions, separate lesions of the dorsal or ventral CA1 were not sufficient
to produce retardation of trace conditioning. It was found that despite the presence of the lesion, animals showed conditioned freezing behavior during the inter-trial interval (ITI), the TI, and the noise CS. It was shown that both dorsal and ventral lesions produced a decrease in freezing response to the contextual as well as trace test. However, the ventral CA1 lesions produced the most profound effect although it was predicted that this effect should have been seen by the dorsal CA1.

The data found in this study are proposed by the authors to show that the dHPC and vHPC contribute to behavior following a gradient, with the ventral CA1 region being the most important to contextual trace fear conditioning. They also argue that their data show that the CA1 region of the HPC is not vital for acquisition of contextual or temporal conditioning.

Misane and colleagues (2005) provide a possible explanation for the unexpected finding that the dHPC was not necessary for acquisition of this task in their study in which they examined the role of the dHPC in auditory and contextual trace fear conditioning using a variety of TIs in animals who received infusions of the NMDA receptor blocker APV into their dHPC prior to training. They discovered that in order for dorsal hippocampal
involvement in trace conditioning to become necessary the TI had to be at least 15 s, which exceeds the 10 s TI used by Rogers et al. (2006). Misane et al. (2005) explains that this may be due to the dHPC playing a critical role in time-dependent processing of noncontingent stimuli.

Yoon and Otto (2007) examined the role of the dHPC and vHPC in trace (with a 30 s TI) and delay fear conditioning using both pre- and post-conditioning lesions. Their study was designed to distinguish between differences in acquisition and expression of conditioning following manipulations in the dHPC versus the vHPC.

This study consisted of two experiments, both assessing differences between the dHPC and vHPC in trace fear conditioning, where separate NMDA lesions were made in each area. The first experiment examined acquisition effects by conducting lesions prior to conditioning while the second experiment examined expression effects by conducting lesions after training. Training in both experiments consisted of a single 10-trial session comprised of a tone CS followed by a 30 s TI terminating in a foot shock US. The timeline of testing varied for each experiment, but both consisted of CS alone presentations in a contextually different chamber. The CR measured was a freezing response as indicated by motion
detectors. Because vHPC lesions have been shown to increase locomotor activity, separate post-testing locomotor activity was measured in an open-field environment for all animals. Control groups consisting of vHPC and dHPC sham lesions were created for both experiments, no significant difference was found between them on any measure, so they were combined in the data analysis.

Animals in the pre-training lesion experiment were tested 24 hr after training. During training, results of this experiment show decreased freezing during the TI and ITI for the vHPC lesion group as compared to both the control and dHPC lesion group. The vHPC lesion group also showed significantly less freezing during the CS as compared to the sham group. There were no differences during the ITI, CS, or TI for the sham and dHPC lesion groups. The testing phase yielded similar results for both the ITI and TI, with the vHPC lesion group showing significantly less freezing than both other groups. During the CS, however, the vHPC lesion and sham groups did not differ but both showed less freezing than the dHPC lesion group. As expected the vHPC group exhibited increased locomotor activity over the other two groups. However no
correlation was found between increased locomotion and decreased freezing.

In the second experiment, animals were trained before undergoing surgery, and were tested after 7 days of recovery. No group differences were found during training, as expected since they had yet to undergo surgery. During testing, the dHPC and vHPC lesion groups exhibited no differences during the ITI, CS, or TI. Both lesion groups showed less freezing as compared to controls for only some of the first three trials during the ITI and TI. No differences in locomotor activity were found for any of the groups.

Results of this study indicate that in trace fear conditioning the vHPC is critical for acquisition, while both the vHPC and dHPC are involved in maintaining the representation of the CS-US association for subsequent expression. The pre-training lesion findings that the dHPC is not necessary for acquisition of trace conditioning are consistent with the previous study by Rogers et al. (2006). However, the finding that lesions of the vHPC disrupt acquisition of trace conditioning is inconsistent with the findings of Rogers and colleagues (2006), who found that lesions of the ventral CA1 region of the HPC.
were not sufficient to cause a significant retardation in trace conditioning. The authors explain that this inconsistency may be due to the present study's inclusion of the CA3 and dentate gyrus, along with the CA1 in their HPC lesion, demonstrating that it may be the CA3 region or the dentate gyrus that is critical to acquisition in this task.

As examined previously, these differences may also be due to differing TIs (30 s vs 10 s) with findings that a TI of at least 15 s is necessary for the dHPC to become critically involved in the task (Misane et al., 2005).

The finding that dHPC lesions had no effect on acquisition in this task was inconsistent with Fendt et al. (2005) who found that lesions of the dHPC prevented acquisition of a fear-potentiated startle trace conditioning task. Yoon and Otto (2007) suggest that differences in the paradigms used are responsible for this discrepancy. They provide evidence by McNish and colleagues (as cited in Yoon & Otto, 2007) that the involvement of the HPC in trace conditioning acquisition may be different in paradigms that use fear-potentiated startle as opposed to freezing. Impairment in trace conditioning acquisition has also been seen after the dHPC
is infused with the NMDA receptor antagonist APV (Misane et al., 2005). The authors argue that when the HPC is lesioned, other brain areas may be compensating for its loss, making CS-US associations in trace conditioning possible. However, when APV is used, the HPC is still able to make some contribution to CS-US processing, perhaps by the action of AMPA receptors, which are not effected by APV, where the AMPA receptors alone are not enough to facilitate acquisition of the trace conditioning task but may be sufficient to hinder compensation from other brain regions.

In the second experiment it was found that both the dHPC and vHPC impair expression of trace conditioned responding. The authors bring up the point that because of the timeline of testing in this experiment it is unjustified to fully explain the results as impairment in expression, because it does not rule out consolidation, storage, or retrieval. The authors explain the finding that animals, including controls, were not freezing to the CS in this task demonstrates that they may have learned that the CS predicts a lack of immediate shock. The authors conclude that the findings of this study in sum support the position that the dHPC and vHPC participate differently in trace fear conditioning.
To examine what specific aspects of trace conditioning are hippocampal dependent, Bangasser, Waxler, Santollo, and Shors (2006) tested whether the HPC is vital for maintaining CS representation activity until US delivery, thereby achieving a sort of temporal contiguity between CS and US. They designed an experiment to test if restoring contiguity in a trace fear conditioning task would enable animals with hippocampal lesions to acquire trace fear conditioning.

In this experiment rats were administered either hippocampal lesions caused by infusion of NMDA or sham lesions. All animals were trained in conditioning boxes with plastic walls and a floor grid with attached shock generator. Contextual cues (wall design, scent, and floor type) in the conditioning boxes varied to differentiate between training and testing phases. Animals were habituated to the chamber for 10 min and to a white noise for 15 s and then underwent 5 training trials in a trace, delay, simultaneous, or contiguous trace conditioning (CTC) procedure. In the trace-conditioning group, a white noise CS was presented for 15 s culminating in a 30 s TI followed by a 2 s shock US. In the delay-conditioning group a 47 s CS was presented co-terminating with the 2 s US. For the simultaneous group a 2 s CS and 2 s US were
presented at the same time. The CTC procedure combined the trace and simultaneous tasks, having a 15 s CS, a 30 s TI, and a simultaneous 2 s CS-US pairing.

After the training day animals were tested. The test procedure for the trace, CTC, and simultaneous procedures all consisted of one 15 s white noise CS. For the delay procedure testing was done with one 47 s white noise CS presentation. Movement was measured in the 30 s period prior to the previous timing of the US in both instances and compared to a pre-CS baseline activity rate.

It was found that lesions of the HPC hindered trace but spared delay fear conditioning. HPC lesioned animals in the trace-fear conditioning procedure showed no increased freezing response over baseline. However, for lesioned rats in the delay-fear conditioning group there was a significant mean difference in conditioned responding during the CS as compared to both their baseline and performance by lesioned rats in the trace-conditioning task.

As predicted it was found that hippocampal lesions did not prevent learning of the CTC. Lesioned animals in the CTC group had more conditioned responding than the lesioned rats in the trace-conditioning task. Importantly, there was not a significant difference in conditioned
responding between lesioned rats and sham operated control rats in the CTC task. It was also found that lesioned rats did not show conditioned responding in the simultaneous conditioning task.

This study demonstrated that the HPC is vital for allowing acquisition of trace conditioning when CS-US contiguity is not present. They found that animals with hippocampal lesions were able to show conditioning in a trace fear-conditioning task only if contiguity had been restored by presenting the CS and US simultaneously after the CS-TI. The authors claim that these finding discredit theories that do not rely on discontiguity as an explanation for hippocampal dependence in trace conditioning. They state that non-contiguity based theories such as the timing theory, which asserts that the HPC is necessary to internally time external cues, are not correct. Possible explanations for the HPC being critically involved in overcoming the discontiguity created with trace conditioning include the argument that it creates a mental bridge between the CS and US either through the use of a Hebbian reverberating circuit or through the use of contextual cueing. It is also possible that the HPC is simply necessary when tasks become increasingly difficult. In this case, animals may have
been able to learn the CTC task simply because it was easier than the trace-conditioning task.

Pharmacological Studies

The HPC and PFC have been shown to play a vital role in the acquisition, consolidation, and expression of memory in variety of different associative learning tasks. Which of these processes is specific to which brain region is still under some debate. With the demonstration that the neurotransmitter acetylcholine (ACh) is necessary for these regionally-specific processes to occur, it was a logical progression to study differentiations in cholinergic function through the use of temporary pharmacological "lesions". We will now discuss the effects of pharmacological agents, such as the NMDA receptor antagonist APV and the protein synthesis inhibitor anisomycin, infused into the HPC on trace and delay conditioning. NMDA is of particular interest because it has been shown to play a critical role in the initial stages of synaptic plasticity (for review, see Segal & Auerbach, 1997)

Wanisch, Tang, Mederer, and Wotjak (2004) examined the acquisition of trace and delay fear conditioning following hippocampal injections of APV or anisomycin.
In their study, they trained mice in fear conditioning chambers and later tested them in a contextually different chamber. All animals had guide cannulae surgically implanted bilaterally into their dorsal HPC prior to training for drug infusion of either APV or anisomycin. Trace conditioning with a 5 s, 15 s, or 60 s TI separating a tone CS from a shock US and delay conditioning with no time gap between the CS and US were carried out in a standard fear conditioning chamber. Testing was later done in a contextually different chamber. The CR measure was the amount of freezing the animal exhibited.

This study consisted of three separate experiments. The first experiment examined the effects of TI duration on conditioning. Mice underwent one day of conditioning in the 5 s, 15 s, 60 s trace paradigms or the standard delay paradigm. They were then tested the next day for both conditioned responding to the tone in the testing chamber and contextual fear conditioning in the conditioning chamber. They found that animals exhibited different amounts of freezing as a function of the TI. There was no difference between the delay conditioning and the 5 s TI. There was a difference however between the delay conditioning and the 15 s and 60 s TIs, with
the delay conditioning showing significantly more conditioned responding than both trace paradigms. The 15 s TI and the 60 s TI were also shown to result in different amounts of conditioned responding, with the 60 s TI showing scientifically less conditioned responding than the 15 s trace. In the context conditioning test, animals were shown to freeze more while undergoing the trace conditioning procedure than the delay conditioning procedure.

The second experiment in this study tested the effects of different doses of APV infused into the dHPC in the delay and 5 s trace conditioning procedures. In this experiment mice were infused with a high or low concentration of APV or vehicle and were then trained and tested for tone CR acquisition in the same manner described in the first experiment. Results demonstrated a dose-dependent reduction in freezing for the trace conditioning group, but not for the delay conditioning group. The higher concentration of APV was found to impair CR acquisition at a higher rate than the lower concentration of APV, with both concentrations retarding CR acquisition more than vehicle injections. In delay conditioning, APV did not significantly effect CR
acquisition. Results demonstrate the importance of early NMDA processes specific to the trace conditioning task.

The final experiment examined the role of protein synthesis in acquisition of trace and delay conditioning. Mice in this experiment had bilateral dHPC infusions of either anisomycin or vehicle and were subsequently trained and tested in the same manner as the second experiment. It was found that anisomycin impaired CR acquisition for trace, but not delay, conditioning, demonstrating the task specific necessity of protein synthesis during trace conditioning.

Wanisch and colleagues (2005) conclude that the HPC plays a role in the early storage of memories associated with the trace conditioning procedure, as demonstrated by its reliance on NMDA receptors and protein synthesis. They explain the findings that heightened context freezing in trace conditioning as compared to that found in delay conditioning may be due to the animal dividing attention during the trace conditioning task between the context and the tone CS, while in delay animals gave their attention more exclusively to the tone CS. In sum, they argue that their data demonstrate that in trace fear conditioning synaptic plasticity in the dHPC may be necessary for the acquisition, storage, and retrieval of memories associated
with the trace conditioning task. The data provided give evidence for the necessary role of NMDA and protein synthesis in the dHPC during the acquisition phase; however further research will be necessary to substantiate the claim that the dHPC is necessary for storage and retrieval.

Microdialysis Studies

In addition to lesion and pharmacological studies of the HPC involvement in trace and delay conditioning, it is necessary to examine non-lesion methods such as microdialysis to gain more complete view of the role of the HPC in Pavlovian conditioning. In one such experiment, Meyer, Allen, and Yokel (1996) used in vivo microdialysis techniques to assess ACh levels in the ventral HPC during delay eyeblink conditioning in rabbits. They were interested in measuring the amount of ACh released during conditioned nictitating membrane reflex in both normal animals as well as those with aluminum injections into their lateral ventricles. The group with aluminum injections in the lateral ventricles was of interest due to findings that they cause retarded cholinergic function in the HPC by decreasing choline acetyltransferase function and well as increasing choline uptake in the HPC.
Rabbits were given intracerebroventricular injections of either aluminum or sodium lactate (for a control) and had microdialysis probes implanted in their right ventral HPC. After recovery they underwent two days of delay conditioning in which they were presented twice daily with 100 paired tone CS and paraorbital shock US presentations. A second pseudoconditioned control group was presented the tone CS alone and the paraorbital shock US alone. Dialysate samples were collected both during a preconditioning baseline and during conditioning.

It was found that rabbits with sodium lactate injections were the only group able to adequately learn the task. The sodium lactate injected group was also found to have significant increases of ACh during the second and third testing session over baseline while the aluminum injected and pseudorandom groups showed no ACh increase across sessions, nor did they learn the CR in the delay conditioning task.

These results show that delay conditioning coincides with an increase in hippocampal ACh release, while an inability to learn the paradigm corresponded with no increase in ACh release. Meyer and colleagues (1996) believe that this demonstrates the critical role of ACh in the HPC in early nicotinic membrane reflex acquisition.
This is supported by the timeline of ACh release and learning in the sodium lactate injected group, which showed the greatest release of ACh during the sessions where the greatest increases in conditioned responding were observed.

Genetic Knockout Studies

Huerta, Sun, Wilson, and Tonegawa (2000) examined the role of NMDA receptors within the CA1 region of the HPC during trace and delay fear conditioning in cell type-specific gene knockout mice. They hypothesized that NMDA receptors within the CA1 pyramidal cells of the HPC are critically involved in encoding temporal memory in mice.

Subjects were 49-78 day old NR1-CA1-KO male mice, which were homozygous for the floxed NR1 gene and heterozygous for the viral Cre recombinase gene. Control mice were homozygous for the floxed NR1 gene. Animals were trained in a fear conditioning chamber which consisted of different colored walls. Testing was done 24 hrs after training in either a chamber comprised of a round basket with bedding on the floors and with gray walls, or with white walls and flooring.
Two training paradigms were used in this study, along with a pseudo-conditioning and a naïve control group. In the trace conditioning paradigm, mice were placed in the training chamber and exposed to 10 sessions of a white noise CS paired with a foot shock US, where the CS and US were separated by a 30 s TI. The delay conditioning paradigm was similar to the trace paradigm, except that the CS and US co-terminated as opposed to being separated by a TI, as they were in the trace conditioning paradigm. Mice from both the trace and delay conditioning groups were tested 24 h after training in one of the testing chambers and were subjected to 10 CS only presentations, each separated by the same ITI as was used in the training session.

They found that the knockout mice have slower CR acquisition during trace conditioning than control mice. Knockout mice froze significantly less than controls during initial trails, although by the third trial there was no difference between the two groups. It was also found that the knockout mice failed to exhibit the CR during the 24 h post-training memory test in the trace conditioning task. In the delay task however, there was no significant difference between the knockout and
control groups. This effect was found both during training and during the 24 h post-training test phase.

The results of Huerta and colleagues (2000) show that NMDA receptors within the CA1 pyramidal neurons are vital for trace, but not delay, fear conditioning. This demonstrates that forming an associative memory where events must be linked across time is NMDA dependent. One possibility put forth to explain the deficit in acquisition of the trace conditioning task in knockout mice is that individual cells in CA1 are be responding specifically to the CS, which sustains the CS during the TI. This continuance of the CS through CA1 cells could allow the association between the CS and the US to be formed, with the temporal overlap of the cell activity in CA1 co-terminating with the US. The authors believe that through NMDA-dependent synaptic plasticity, the representation of the CS is "entrained" into cell ensembles of the CA1. It is through this process that an enhancement in the covariance of cell ensemble responses takes place.

To further examine the role of the specific areas of the HPC in trace conditioning Kishimoto, Nakazawa, Tonegawa, Kirino, and Kano (2006) examined CA3-NR1 knockout mice in trace and delay eyeblink conditioning.
In the trace conditioning procedure, CA3-NR1 knockout mice, and homozygously floxed-NRI controls were given a 10 day conditioning phase in which a tone CS was followed by a 500 ms TI terminating in a periorbital shock US. For the delay conditioning procedure, the tone CS was followed immediately by the periorbital shock US. CR timing was measured using electromyogram (EMG) methods.

The authors first replicated findings that bilateral HPC lesions (using ibotenic acid) impair trace, but not delay, eyeblink conditioning. Knockout mice and controls where divided into three groups and underwent training and subsequent 4 day extinction trials in either a trace conditioning procedure (10 days), a delay conditioning procedure (7 days), or a pseudoconditioning procedure.

It was found that knockout mice had adequate acquisition of the trace conditioning task, but showed impaired ability to extinguish during the extinction phase. Although they were able to demonstrate acquisition during trace conditioning, knockout mice were found to be unable to properly time their CRs during the training phase. During the delay conditioning task, knockout animals were found to be similar to control animals, demonstrating the ability to both acquire properly timed
acquisition of the task and show extinction equivalent with that of control animals.

These results indicate that pyramidal cells in the CA3 region of the HPC are necessary for CR timing and extinction in trace conditioning, while they are not necessary for these aspects of delay conditioning. Kishimoto and colleagues (2006) believe that timing deficits are due to adaptively timed CR acquisition requiring recruitment of both CA1 and CA3 memory networks. With the CA3 network missing, animals are still able to rely on CA1 networks to activate the memory trace well enough to allow acquisition of the task, but without the precision that a fully intact hippocampal network would allow. They further explained the knockout animals’ inability to extinguish to the trace conditioning task being a result of retarded “internal inhibition”. This is demonstrated in the animals’ persistence to show conditioned responding to a no longer predictive cue as well as their increased CR rate over control animals at CS onset during the trace conditioning task.

Developmental Studies

Moyer and Brown (2006) tested the effect of normal aging on trace and contextual fear conditioning. Aging has
been shown to cause deficits in tasks requiring the medial temporal lobe, which include the HPC and the perirhinal cortex. To explore this, they tested rats in trace fear conditioning, which requires an intact HPC, and delay fear conditioning, which does not require an intact HPC.

In this experiment, rats were age-matched into 4 groups: an adult group (3-6 months of age), an early middle-aged group (8-12 months), a late middle-aged group (16-20 months), and an aged group (24-33 months). During training, animals were placed in a fear conditioning operant chamber. They were given 10 tone-trace-shock trials (each trial consisting sequentially of a 15 s tone CS, a 30 s TI, and a 1 s foot shock US). Day two of the experiment consisted of a tone-alone test. Animals were brought to a contextually different hexagonal Plexiglas chamber and presented with a 6 min tone CS. During day three, animals were tested for context conditioning in the fear conditioning operant chamber with no stimuli for 10 min after which they were removed.

A subset of the rats (early middle aged, late middle aged, and aged) that didn't learn the trace fear conditioning paradigm were tested in a short-delay fear conditioning task 1-4 weeks after the tone test. This task was very similar to the initial trace fear conditioning
task previously trained, however the TI was changed to 0 s and the mean ITI was changed to 3 min. As a control, an additional group of naïve aged rats was added at this point and tested in a long-delay fear conditioning task. In this task, a 46 s noise CS was immediately followed by a 1 s food shock US, making it the same amount of time as the trace fear conditioning task.

It was found that in trace fear conditioning aged rats froze less to the onset of the cue as compared to adult, early middle-, and late middle-aged rats. This same pattern was seen during the post-CS period. Aged rats had fewer expressions of fear during all time blocks of the session as compared to all other groups. In the contextual conditioning test there was a difference in percent of freezing expressed as a function of age, with aged rats showing the lowest levels of freezing in this context. Aged rats froze less than adult and early middle-aged rats. It was also reported that late middle-aged rats have fewer expressions of freezing than early-middle aged rats.

It was also found that there were age specific differences in the timeline of freezing behaviors. In adult and early middle-aged rats there was a constantly high level of freezing shown throughout testing, in late middle-aged rats a high level of freezing was not seen
until the second minute into the task. The pattern was different with aged rats that never achieve high levels of conditioned freezing.

In the short-delay fear conditioning task it was found that aged rats showed a significant difference in conditioned freezing as compared to their performance in the trace fear conditioning task. During the subsequent tone test task it was found that level of CR significantly increased in the delay task and compared to the trace task across time. In addition, aged rats showed improved conditioning in the short-delay conditioning task compared to the trace conditioning task. However, they did not show improvement in the contextual conditioning test.

It was found that aged rats in the long-delay task were able to show significant conditioned responding to the CS, although they had difficulty acquiring the trace conditioning task. However, there were no age related differences in foot shock sensitivity or baseline freezing activity levels.

Results of this study show that aged animals show retarded learning in trace and contextual fear conditioning, which are both tasks shown to rely on the HPC and/or perirhinal cortex. It was also shown aged animals had normal learning in both short- and long-delay
conditioning, which are not reliant on the HPC or perirhinal cortex.

The authors believe that these deficits are a function of age related changes in the HPC and perirhinal cortex. This is supported by the systematic decrease in performance on only trace and contextual conditioning tasks shown as the animals age. Because aged animals were able to perform optimally on long-delay procedures it is believed that it is due to the TI that the animals have difficulty learning the task and not time between CS onset and US onset. These deficits were also found to not be a result of sensorimotor, fear conditioning, or sensitivity deficits.

Overall, these findings indicate that the HPC plays an important role in trace conditioning. The lesion studies demonstrated that dHPC lesions do not affect contextual conditioning, while they do retard fear-potentiated startle response (Fanselow et. al, 2005). In a different paradigm, measuring freezing during a shorter TI, the ventral CA1 region was shown to be most important during contextual trace fear conditioning, while neither the dorsal CA1 region nor the ventral CA1 region of the HPC were individually critical to trace fear conditioning (Rogers et al., 2006). Impairment in trace conditioning
acquisition was demonstrated however after the dHPC is infused with the NMDA receptor antagonist APV, but only with TIs exceeding 15 s (Misane et al., 2005). Another study implicated that the vHPC is critical for acquisition while both the vHPC and dHPC are involved in maintaining the representation of the CS-US association for subsequent expression in trace conditioning. The fact that these finding contradict previously discussed results opens the possibility that it may be the CA3 region or the dentate gyrus that is critical to acquisition in this task (Yoon & Otto, 2007).

It was also demonstrated that the HPC is critical for acquisition of trace conditioning when CS-US contiguity is not present (Bangasser et al., 2006). Animals with hippocampal lesions were able acquire adequate trace conditioning only if contiguity had been restored by presenting the CS and US simultaneously after the CS-TI.

The role of the HPC in trace and delay conditioning was then extended with the inclusion of a pharmacological study which concluded that in trace fear conditioning NMDA dependent synaptic plasticity and protein synthesis in the dHPC may be necessary for the acquisition, storage, and retrieval of memories associated with the trace conditioning task (Wanisch et al., 2005). The specific
role of the neurotransmitter ACh was then examined in a study which found that adequately acquiring the CR in delay conditioning coincides with an increase in hippocampal ACh release, while an inability to learn the paradigm corresponded with no increase in ACh release (Meyer et al., 1996).

A study using genetic knockout mice indicated that pyramidal cells in the CA3 region of the HPC are necessary for CR timing and extinction in trace, but not delay, conditioning (Kishimoto et al., 2006). Consistent with damage to the HPC normal aged animals exhibit retarded learning in trace and contextual fear conditioning, but had normal learning in non-hippocampal dependent learning such as short- and long-delay conditioning.

In sum these studies demonstrate that the HPC is important in trace, but not delay, conditioning. However, the role of the HPC in trace conditioning is multifaceted, as it depends on not only which part of the HPC is involved but also on specific aspects of the trace conditioning task, such as CR type, TI duration, and contiguity.
CHAPTER THREE

THE ROLE OF THE PREFrontAL CORTEX IN TRACE AND DELAY PAVLOVIAN CONDITIONING

Introduction

The medial prefrontal cortex (mPFC) is comprised of many distinct regions (Uylings, Groenewegen, & Kolb, 2003) including the prelimbic cortex, infralimbic cortex, and anterior cingulate cortex (ACC), each of which have been implicated in the various aspects of trace conditioning. The mPFC has also been implicated as a source of attention or awareness for numerous tasks. The role of the mPFC in Pavlovian conditioning is still not entirely clear, although many recent advances have been made demonstrating its specific role in trace conditioning.

It has been demonstrated that bilateral aspiration lesions of the caudal mPFC, specifically the supragenual portion of the anterior cingulated cortex, retarded acquisition of a trace eyeblink CR, without impairing delay eyeblink conditioning acquisition (Kronforst-Collins & Disterhoft, 1998). Lesions of the rostral mPFC (i.e. the dorsal anterior cingulated cortex and prelimbic cortex), however, impaired extinction but not acquisition of trace conditioning. The finding that aspiration lesions of the
caudal mPFC prevent acquisition of trace conditioning has been replicated by Weible, McEchron, and Disterhoft (2000), suggesting that the caudal mPFC may function as a storage site for the association in trace conditioning or may provide an essential link between other critically involved regions.

McLaughlin, Skaggs, Churchwell, and Powell (2002) tested the role of the PFC, specifically the prelimbic cortex and ACC, in trace and delay eyeblink conditioning using a variety of CS and ISI durations. They conducted a series of experiments testing the role of the PFC in trace and delay eyeblink conditioning, using a tone CS paired with a pariorbital shock US measuring both conditioned eyeblink and conditioned changes in heart rate.

They found that lesions of the prelimbic cortex had only a moderate effect on eyeblink and heart rate with a 100 ms CS during trace conditioning acquisition as compared to delay conditioning. This inability of prelimbic cortex lesions to disrupt trace conditioning was not dependent of TI length, as both 500 ms and 1,000 ms TI durations were equally unaffected. However, prelimbic cortex lesions did retard acquisition of trace conditioning with a 500 ms CS, as compared to delay conditioning. Although, with continued training animals
were able to overcome lesioning effects and perform at sham levels.

They then tested the effects of ACC lesions in trace conditioning and found that while there were lesion induced differences in heart rate CR, there was no deficit in eyeblink CR for rabbits with ACC lesions. This finding is inconsistent with previous research (see Kronforst-Collins & Disterhoft, 1998; Weible et al., 2000) showing that ACC damage caused a profound deficit in trace conditioning using a nictitating membrane CR.

The role of the ACC in trace and delay conditioning was further examined by Han and colleagues (2003). They tested the role of attention, through the use of visual distraction, in mice with ACC lesions in both trace and delay fear conditioning. Visual distraction was found to selectively impair trace conditioning, indicating that trace conditioning requires a greater deal of attention than does delay or contextual conditioning. It was also found that c-fos levels were significantly greater in the ACC for animals trained in trace conditioning as compared to the other conditioning groups. Also, it was found that ACC lesions retard trace conditioning acquisition, while not effecting delay or contextual conditioning. Lesions of
the primary visual cortex were found to not cause impairment in trace, delay, or context conditioning.

Takehara-Nishiuchi, Kawahara, and Kirino (2005) examined the role of NMDA receptors within the PFC in the acquisition and early consolidation phases of both trace and delay eyeblink conditioning in rats. They examined the role of the mPFC by using reversible methods to assess specific roles of NMDA dependent synaptic plasticity using a GABA\(_A\) receptor agonist and a NMDA receptor antagonist. The subjects were 9-week-old male Wistar rats surgically implanted with bilateral guide cannulae in the mPFC for intracerebral microinfusion. Rats were infused with the GABA\(_A\) agonist muscimol HBr, APV, or vehicle. The time of drug infusion was dependent on group; a pre-conditioning group received infusions 10 min before, a post-conditioning group received infusions immediately after, and a 3-h group received injections 3 hr after conditioning sessions. After recovery, animals were tested for spontaneous baseline eyeblink rates. All rats then underwent 100 trials per day in either a trace conditioning or a delay conditioning paradigm. In both paradigms, a tone CS was paired with pariorbital shock US to the left upper eyelid. In the trace group there was a
500 msec time gap between the CS and US, whereas in the delay task the US and CS co-terminated.

It was found in the pre-conditioning group that inactivation of the mPFC by muscimol during trace eyeblink conditioning trials retarded acquisition and moderately decreased post-learning expression of the CR. Infusion of APV during trace eyeblink conditioning trials significantly impaired CR acquisition, while sparing post-learning CR expression. Muscimol was also found to significantly decrease spontaneous eyeblink rates. In the post-training group, both APV and muscimol retarded CR acquisition when it was given immediately after trace conditioning. However, this effect was not found in the 3-h group when APV and muscimol were given three hours after training. It was also found that muscimol infusion did not effect CR acquisition for any group.

Results of this study suggest that the mPFC is crucial for acquisition and early consolidation of CRs in trace, but not delay, eyeblink conditioning. Demonstrate that synaptic modification occurring during and after training, necessary for memory consolidation, are mPFC dependent.

A similar impairment in trace conditioning performance was found with mPFC NMDA blockade 1 or 2 weeks
after acquisition of trace conditioning, although this 
impairment was not found when NMDA blockade occurred 1 day 
or 3 to 4 weeks after acquisition (Takehara-Nishiuchi, 
Nakao, Kawahara, Matshuki, & Kirino, 2006). This implies 
that NMDA receptors in the mPFC play a time dependent role 
in consolidation and/or retrieval of trace conditioning.

Simon, Knuckley, Churchwell, and Powell (2005) 
examined the effects of post-acquisition lesions of the 
mPFC at multiple time points after acquisition (24 h, 1 
week, 2 weeks, and 1 month post training). They discovered 
that lesions at all of these time points caused impairment 
of later performance of trace conditioning. However, this 
detriment was primarily only evident on the first day of 
retesting as animals began performing closer to sham 
controls by the second day of retesting.

Combined this data gives strong support for the vital 
role of NMDA receptors in the mPFC while acquiring the 
trace conditioning task. This NMDA dependent effect is 
most likely do to NMDA’s role in memory formation through 
synaptic plasticity. The authors argue that the finding 
that muscimol inhibited post-learning expression, summed 
with the previously mentioned findings, leads to the 
conclusion that the mPFC plays a role in acquisition, 
consolidation, storage, and retrieval in trace
conditioning, with an emphasis on late memory processes. Together these findings provide evidence for the vital role of the mPFC in acquisition trace, but not delay, Pavlovian conditioning.

If the mPFC is to be demonstrated as a location for the stored association in trace conditioning as is suggested by Weible and colleagues (2000) then more information about its role later in performance must be acquired.
CHAPTER FOUR
THESIS PROPOSAL

Introduction

Pavlovian conditioning is a form of associative learning in which an originally neutral CS becomes associated, through pairing, with a biologically meaningful US. This procedure can be carried out using a variety of different temporal CS-US arrangements. The fastest learning typically comes from arranging the CS so it begins prior to and then coterminates with the onset of the US, as is the case with delay conditioning. In trace conditioning, which is more difficult to learn, the CS offset precedes the US onset by a fixed interval of time. Unlike delay conditioning, trace conditioning requires cognitive processes including attention and declarative memory, which depend on the mPFC and HPC, respectively. The HPC has been shown to play a vital role in the acquisition of trace, but not delay, conditioning tasks (Bangasser et al., 2006; Fendt et al., 2005; Misane et al. 2005; Takehara-Nishiuchi et al., 2005; Takehara-Nishiuchi et al., 2006; Yoon & Otto, 2007). It has also been demonstrated that the HPC is necessary for consolidation of trace, but not delay, conditioning as is
evidenced by impaired trace conditioning performance when the HPC is damaged one day after acquisition (Kim et al., 1995; Quinn et al., 2002). However the HPC is not necessary for post consolidation performance of trace or delay conditioning. Specifically, it was found that hippocampal lesions made one month after acquisition of trace conditioning had no detrimental effect on performance, although lesions made one day after acquisition caused a selective impairment of trace, but not delay, conditioning (Kim et al., 1995).

The mPFC has similarly been implicated in the acquisition (Kronforst-Collins & Disterhoft, 1998; Weible et al., 2000) and consolidation (Takehara-Nishiuchi et al., 2006) of the trace, but not delay, conditioning. However, unlike the HPC, the mPFC appears to continue to play a role after consolidation is complete in trace conditioning. This demonstrated by the finding that mPFC lesions made anywhere from one day to one month post-acquisition impaired initial retesting performance in trace conditioning (Simon et al., 2005).

It has been previously reported that basal forebrain cholinergic lesions using the selective cholinergic immunotoxin 192 IgG-saporin (SAP) cause a selective impairment in the acquisition of an appetitive trace, but
not delay or long delay, conditioning task (Butt et al., 2007). The use of a variation of standard delay conditioning, a paradigm known as long-delay conditioning controlled for the possibility that the lesion-induced impairment in trace conditioning was due to an inability to form associations between stimuli separated by relatively long ISIs. Cholinergic lesions had no effect in a long-delay protocol where the ISI was matched to that used in the trace conditioning task. Instead, the selective trace conditioning impairment observed following lesions of the basal forebrain appear to result from an inability to bridge the temporal gap separating the CS and US, perhaps by disrupting the maintenance of the representation of the CS during the TI.

Butt and colleagues (2007) also tested the role of the cholinergic basal forebrain in the mediation of attention, which was challenged by presenting a visual distracter during acquisition of trace, delay, and long-delay conditioning in rats with basal forebrain cholinergic lesions. The presentation of a visual distraction exacerbated lesion-induced impairments in trace conditioning, but had no effect on animals with cholinergic lesions in the delay or long-delay conditioning paradigms. These findings are consistent with
reports showing that damage to the PFC or HPC cause a dissociation of impairment between trace and delay conditioning. The basal forebrain cholinergic system projects to both of these regions via the medial septum (MS), which sends cholinergic projections to the HPC, the diagonal band of Broca, which projects to cingulate cortex, and the nucleus basalis magnocellularis (NBM), which projects to neocortex including mPFC (McKinney, Coyle, & Hedreen, 1983). The fact that many of these structures are involved in the successful acquisition of trace, but not delay conditioning, and that these brain regions are modulated by the excitatory neurotransmitter ACh, suggests that this neurotransmitter may be selectively involved in mediating trace conditioning.

Consistent with this argument, performance in a trace eyeblink conditioning paradigm was not affected by lesions of the MS made after animals acquired the CR. In contrast, MS lesions made prior to training significantly impaired acquisition in the trace conditioning paradigm. Interestingly, this lesion-induced impairment in trace conditioning acquisition was attenuated by administration of the cholinergic agonist drug carbachol (Fontán-Lozano, Troncoso, Múnera, Carrión, & Delgado-Garcia, 2005).
The role of ACh in trace conditioning has also been examined using cholinergic antagonist drug treatments in aversive trace conditioning paradigms. For example, high doses of the cholinergic antagonist scopolamine hydrochloride (HCl) were found to completely block trace conditioning acquisition, although animals could subsequently learn the task once the drug had cleared from their system (Kaneko & Thompson, 1997). Similar findings were also obtained in an appetitive trace conditioning task where scopolamine disrupted performance (Seager, Asaka, & Berry, 1999). Although these studies clearly indicate cholinergic involvement in trace conditioning, the systemic injection of scopolamine affects the entire brain and therefore these studies lack precision with respect to the anatomical location of ACh’s critical action in trace conditioning.

The specific role of ACh in the HPC and mPFC in trace and delay conditioning has not yet been directly determined. Assessing cholinergic function in the HPC and mPFC during the performance of trace and delay conditioning can therefore contribute significantly to our knowledge of the basal forebrain cholinergic system’s involvement in trace and delay conditioning.
The goal of the current experiment was to examine the pattern of ACh release in the mPFC and HPC during performance in trace and delay appetitive conditioning. Based on reports of enhanced prefrontal ACh release in attention-dependent tasks (Himmelheber, Sarter, & Bruno, 2001) and on findings that the mPFC is important for trace conditioning performance (Simon et al., 2005), it was predicted that testing-induced ACh efflux will be greater in the mPFC during trace conditioning performance than during delay conditioning performance. However, based on reports showing that post-consolidation hippocampal lesions have no effect on trace or delay conditioning performance (Bangasser et al., 2006; Kim et al., 1995; Kronforst-Collins & Disterhoft, 1998), it was hypothesized that testing-induced ACh efflux in HPC may be greater during performance of trace conditioning than during performance of delay conditioning in the current experiment, although in over-trained animals HPC involvement may be minimal.
CHAPTER FIVE

THESIS EXPERIMENT

Methods

Guidelines for Animal Use

Subjects were cared for according to the requirements set by the Society for Neuroscience, the American Psychological Association, the California State University, San Bernardino (CSUSB) Animal Care and Use Committee, and the "Guide for the Care and Use of Mammals in Neuroscience and Behavioral Research" (National Council, 2003).

Animals

Subjects were 13 male Long-Evans rats (Harlan, Indianapolis, IN) weighing 300-350 g. upon arrival. Animals were housed in an environment maintained at 21-23 °C with ad libitum water on a 12-hour reverse light cycle (lights off 06:00 h). Beginning one week prior to pretraining, animals were maintained at 85% of their normal body weight for the duration of the experiment.

Apparatus

Training and testing was conducted in individual computer-controlled, sound-attenuating operant chambers (Coulbourn Instruments, Allentown, PA) equipped with a
speaker for presenting white noise (80 dB) and equipped with a light located over the food magazine. US presentations consisted of the delivery of a single sucrose pellet (45 mg; MedAssociates, Lancaster, NH) into a food magazine (MedAssociates, Lancaster, NH) located at floor level. Snout entries into the food magazine were assessed using photobeam response detectors (MedAssociates, Lancaster, NH) located inside the food magazine. A 1 W white light located at the top of the chamber provided ambient illumination. The presentation of the white noise CS, light CS, and the delivery of the sucrose pellet US was controlled via computer interface (WINLINC, Coulbourn Instruments, Allentown, PA). The operant chambers were each modified with the addition of 12 in aluminum wall extensions, an aluminum roof, and an extended front Plexiglas door to allow room for the microdialysis swivel at the top of the chamber. A video surveillance camera and a photo-beam movement detection device were be used for additional behavioral assessment. The side wall of each chamber had a small hole to allow passage of microdialysis tubing traveling from a small infusion pump (Bioanalytical Systems Inc, West Lafayette, IN) located outside the sound-attenuating chamber housing.
each unit, via a 5 channel liquid swivel (Instech Laboratories, Plymouth Meeting, PA).

Surgery

After pretraining, rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.; Sigma Aldrich, St. Louis, MO) and surgically implanted with two MAB 4 series microdialysis probe guides fitted with sterile, stainless steel temporary stylets (SciPro, Inc., Sanborn, NY) targeting the right HPC (AP: -5.8, ML: +5.0, DV: -2.5, from dura) (see Pych, Chang, Colon-Rivera & Gold, 2005) and the right PFC (AP: +2.7, ML: +0.8, DV: -2.5, from dura) using established stereotaxic coordinates (Paxinos & Watson, 2004). Animals will be then given i.p. injections of analgesic (Ketaprofin, 2 mg/kg, s.c; Western Medical Inc, Anaheim, CA) and antibiotic (Baytril, 2.5 mg/kg s.c.; Western Medical Inc, Anaheim, CA) and allowed 36 hrs for recovery.

General Procedure

One day prior to the beginning of training, animals were tether-trained and magazine-trained in the testing chamber for one hour. They then underwent the pretraining phase that lasted for 15 days and consisted of a mixed trace/delay conditioning protocol. Each daily session of this protocol consisted of a 45 min stimulus-free baseline
period to allow acclimatization to the test chamber, followed by 30 trials of either the trace or the delay conditioning paradigm, followed by 30 trials of the other conditioning paradigm (trace or delay conditioning). The sequence of the trace and delay conditioning blocks was pseudorandomly determined across days. Different discriminative CS stimuli were used for the trace and delay conditioning tasks and were counterbalanced across animals to control for stimulus type. The CS for the delay or trace conditioning trials was either a 10 s white noise or a 10 s light. In the trace conditioning paradigm, the CS was followed after a 10 s TI (TI) by a sucrose pellet US, with average ITI of 40 s (range 20 - 60 s). In the delay conditioning paradigm, the CS was followed immediately by a sucrose pellet US, with an average ITI of 50 s (range 40 - 70 s). In order to ensure habituation to the microdialysis tether, animals were tethered throughout the pretraining phase.

Conditioned responding was assessed by measuring the duration of time spent with the snout in the food magazine during the 10 s CS presentation, 10 s TI, and 10 s pre-CS baseline period (each 10 s period consists of five 2 second bins). For the delay conditioning task learning was measured by calculating CS nose poke duration - preCS nose
poke duration and for the trace conditioning task learning was measured by calculating TI nose poke duration - preCS nose poke duration for the trace task.

Only animals demonstrating adequate learning at the end of pre-training underwent microdialysis testing. Adequate learning was assessed using a conditioning ratio for each task. For the delay conditioning task this was calculated as the total duration of CS responses/ the sum of the total duration of CS responses and total pre-stimulus response averaged across the last three days of the task (including the test day). For the trace conditioning task this was calculated as the total duration of TI responses/ the sum of the total duration of TI responses and total pre-stimulus response averaged across the last three days of the task. Adequate learning was defined as performance with a minimum conditioning ratio of 0.60. This criteria represents a CR level that is at least 50% greater during the CS interval than during the pre-stimulus period for delay conditioning, or at least 50% greater during the TI than during the pre-stimulus period for trace conditioning.

Thirty-six hours after recovery from surgery, animals meeting these performance criteria underwent one day of dual probe microdialysis sampling during behavioral
testing. Prior to being placed in the testing chamber, rats were tethered and their stainless steel guide stylets were removed to allow insertion of a 2 mm microdialysis probe into their mPFC probe guide, and insertion of a 3 mm microdialysis probe into their HPC probe guide (Models 14.2 and 14.3 PES, respectively, SciPro Inc., Sanborn, NJ). Both probes were continuously perfused (0.5 µl/min) with artificial cerebrospinal fluid (aCSF) containing 50 nM of the acetylcholinesterase inhibitor neostigimine bromide (148 mM NaCl, 4 mM KCl, 1.2 mM CaCl₂, 1.2 mM MgCl₂, 50 nM neostigimine bromide; pH 6.8). The necessity of using neostigimine bromide was demonstrated in pilot experiments, where ACh efflux levels were found to be below the threshold for detection using HPLC and electrochemical detection methods. This finding was consistent with data reported by Chang, Savage, and Gold (2006), who also demonstrated the need to include acetylcholinesterase inhibitor in the aCSF in order to detect basal ACh levels. Unpublished observations from our lab show that neostigimine bromide does not influence behavior in the combined delay/trace conditioning task.

Rats underwent a 3 hr quiet period in the testing chamber in order to stabilize neurotransmitter release before beginning behavioral testing and microdialysis.
sampling. This equilibration period was followed by a 1 hr stimulus-free baseline period, where microdialysis samples were collected, followed immediately by the onset of the delay/trace conditioning procedure. To ensure no neurotransmitter carryover from one conditioning protocol to the next, a 1 hr stimulus-free period, was separated the delay and trace conditioning procedures during this microdialysis testing phase of the experiment.

Dialysate samples were collected every 30 min throughout the baseline and testing phases. Samples were quantified for ACh levels using high performance liquid chromatography (HPLC) with electrochemical detection (Bioanalytical Systems Inc, West Lafayette, IN). ACh release was expressed as a percentage of baseline release by dividing testing-induced peak area by the mean baseline peak area for the two 30-min baseline samples collected prior to each conditioning protocol.

Statistical Analyses

Pretraining behavioral data was analyzed using a within-subjects one-way analysis of variance (ANOVA) for both the trace and delay testing blocks. The dependent variable (DV) for this measure was nose poke difference scores, calculated as CS nose poke duration - preCS nose poke duration for the delay task and TI nose poke duration
- preCS nose poke duration for the trace task. These difference scores were derived from cumulative responding across the 30 conditioning trials in each paradigm. The CS duration scores in the delay conditioning task as well as the TI duration score in the trace conditioning task included only the last 4 s of the 10 s CS or TI and were compared to a comparable 4 s pre-stimulus score in order to measure adaptive responding in both tasks. ACh data was analyzed using a two-way (conditioning task x brain region; HPC and mPFC) within-subjects ANOVA. Specific planned comparisons were analyzed using paired t-tests.

**Histology**

Upon completion of behavioral testing, rats were killed by a lethal dose of sodium pentobarbital (80 mg/kg, i.p.; Sigma, St. Louis, MO) followed by cardiac perfusion with 0.9% saline for 5 min and 10% formalin for 30 min. Brains were extracted and placed a 25% sucrose solution for 48 hrs prior to freezing and sectioning. Sections (60 μm) were stained with thionin and examined to verify probe placements.
CHAPTER SIX
RESULTS AND DISCUSSION

Results

Probe Placement Verification

For verification of placement of microdialysis probes, brains were sectioned and stained (see Figure 1). All probe placements were determined to be within the boundaries of the mPFC and HPC, respectively. The mPFC probe placements were primarily within the prelimbic cortex and cingulate cortex. Hippocampal probe placements were located within the hippocampal formation, including CA1, CA2, and CA3 subregions.

Behavioral Results

Seven animals demonstrated adequate learning as defined by a conditioning ratio \( \geq 0.60 \) for both conditioning tasks (see Chapter 5); data from these animals are therefore included in the following analyses. Two t-tests were used to compare counter-balanced cue conditions within each task for the animals receiving a light cued trace and white noise cued delay \((n = 4)\) and animals receiving a white noise trace and a light cued delay \((n = 3)\); i.e. trace conditioning: light CS vs white noise CS and delay conditioning: white noise CS vs light
Figure 1. Probe Placement. Probes targeting mPFC occupied prelimbic and cingulate cortex; probes targeting HPC occupied all subregions of HPC.
There were no cue differences in noise poke duration in the trace conditioning task between the two types of cues during either the TI or during the CS. It was found that there was a difference based on cue type during the delay conditioning task with more responding occurring when the delay CS was a white noise; two-tailed t-test yielded, \( t(5) = 3.24, p < .05 \). This is likely due to the fact that light cues predicting food cause conditioned rearing, which will take away from time approaching the food cup (Holland, 1980). However, neurochemistry results demonstrated that there was no significant difference in ACh efflux between the two cue types in either the trace or delay conditioning task.

Analyses of the behavioral data from all rats indicate that they learned the CR in both tasks. Behavioral data in the trace conditioning task indicates that nose poke duration difference scores increased across pretraining and testing days; ANOVA yielded a significant day main effect, \( F(15, 90) = 5.18, p < .001 \) (see Figure 2a). An increase in nose poke duration difference scores also occurred across days in the delay conditioning task; ANOVA yielded a significant day main effect, \( F(15,90) = 5.29, p < .001 \) (see Figure 2b). There were no significant
Figure 2. Conditioned Responding Acquisition. Data show mean CR duration difference scores (± SEM) across acquisition training (Day 1-15) and on the day of microdialysis testing (Day 16) in the trace conditioning.
(2a; TI; black circles) and delay conditioning (2b; Delay CS; black diamonds) paradigms. Data show the duration of response during the last 4 seconds of the CS and TI, respectively. Across pre-training rats acquired the CR in both behavioral tasks.
mean differences in performance between the day of microdialysis and the final day of pretraining for either the trace \((t(6) = 1.96, p > .05)\) or delay \((t(6) = 2.06, p > .05)\) conditioning task.

Visual analysis of the pattern of behavioral responding demonstrates that rats developed adaptive conditioned responding in both behavioral tasks. In the trace conditioning task, minimal conditioned responding occurred during the CS itself, where responding during the CS can be viewed as being premature and thus non-adaptive (see Galvez, Weible, & Disterhoft, 2007). Instead, the duration of responding in the food cup progressively increased across the TI and reached a maximum just before US presentation (see Figure 3a). A similar pattern can be seen in delay conditioning where responding increased progressively throughout the CS and reached a maximum just before US presentation (see Figure 3b).

**Neurochemistry Results**

Comparisons of percent of baseline ACh release in mPFC and HPC during trace and delay conditioning revealed a significant main effect of both task and brain region; ANOVA yielded a task effect of \(F(1,6) = 8.29, p < .05\) and a brain region effect of \(F(1,6) = 6.96, p < .05\) (see...
Figure 3. Adaptive and Non-adaptive Responding. Data show responding in the trace conditioning (3a) and delay conditioning (3b) paradigms on the final day of training (day 15). Data show the average duration of conditioned approach responding in the food cup (difference from baseline rates of responding) across 2 second intervals during the 10 s CS and during the TI in the trace conditioning paradigm. Note the progressive increase in duration of adaptive responding during the CS in the delay paradigm, where responding reaches its peak just before the US is delivered. In trace conditioning, non-adaptive responding during the CS is modest and adaptive responding during the TI peaks at the time of US delivery the US.
Figure 4). Collapsed across brain regions, ACh efflux was greater during trace conditioning (m = 159%, sd = 0.41) than during delay conditioning (m = 103%, sd = 0.47). Collapsed across task, ACh efflux was greater in the mPFC (m = 149%, sd = 0.53) than the HPC (m = 113%, sd = 0.35). There was no significant brain region by task interaction.

As shown in Figure 4, during trace conditioning the mPFC had greater ACh efflux compared to hippocampal ACh efflux; a one-tailed paired-samples t-test yielded t(6) = 2.61, p < .05. In contrast, during delay conditioning the mPFC did not show greater ACh efflux compared HPC ACh efflux; a one-tailed paired-samples t-test yielded t(6) = 1.16, p > .05. ACh efflux in the mPFC was greater during trace conditioning than during delay conditioning; a one-tailed paired-samples t-test yielded t(6) = 2.69, p < .05. Similarly, ACh efflux in the HPC was greater during trace conditioning than during delay conditioning; a one-tailed paired-samples t-test yielded t(6) = 2.68, p < .05.

Comparisons of the testing-induced ACh efflux to ACh efflux during its comparable baseline for each brain region during each task revealed that the during trace conditioning the mPFC had a significant increase in testing induced ACh release over baseline release; a one-tailed paired-samples t-test yielded t(6) = 3.05, p < .05.
Figure 4. Acetylcholine Efflux. Mean (± SEM) testing-induced ACh release (percentage of baseline release) in the PFC and HPC during the trace and delay conditioning paradigms. ACh efflux during trace conditioning (left) was significantly greater than during delay conditioning (right). Testing-induced ACh efflux was also significantly increased over baseline efflux in both brain regions during trace conditioning (*) but not during delay conditioning. Direct comparisons of brain regions demonstrated that ACh release in the mPFC during trace
conditioning was greater than mPFC ACh release during delay conditioning. ACh release in mPFC was also greater than in the HPC ACh release during both trace and delay conditioning. ACh release in the HPC was greater during trace conditioning than during delay conditioning.
Similarly, during trace conditioning the HPC had a significant increase in testing induced ACh release over baseline release; a one-tailed paired-samples $t$-test yielded $t(6) = 2.21, p < .05$. In contrast, during delay conditioning the mPFC did not have a significant increase in testing induced ACh release over baseline release; a one-tailed paired-samples $t$-test yielded $t(6) = 0.40, p = .35$. Also, during delay conditioning the HPC did not have a significant increase in testing induced ACh release over baseline release; a one-tailed paired-samples $t$-test yielded $t(6) = -0.44, p = .34$.

Comparisons of percent of baseline ACh release in mPFC and HPC during trace and delay conditioning in the six animals that did not adequately learn both tasks (see Chapter 5) revealed no significant main effect of either task ($F(1,5) = 0.20, p > .05$) or brain region ($F(1,5) = 0.27, p > .05$) and no interaction ($F(1,5) = 0.13, p > .05$; see Figure 5). These animals had an average trace conditioning ratio 0.55 and an average delay conditioning ratio of 0.63 while animals who were considered to have adequately learning had an average trace conditioning ratio of 0.69 and an average delay conditioning ratio of 0.68.
Figure 5. Acetylcholine Efflux in Non-learners. Mean (± SEM) testing-induced ACh release (percentage of baseline release) in the PFC and HPC during the trace and delay conditioning paradigms.
Discussion

The goal of the current experiment was to examine the pattern of ACh release in mPFC and HPC during performance in a combined trace and delay Pavlovian conditioning task. As hypothesized, it was found that ACh levels in the mPFC were greater during performance of trace conditioning than during performance of delay conditioning. ACh levels in the HPC were also found to be greater during performance of trace conditioning than during performance of delay conditioning, although HPC ACh levels were lower than mPFC levels during trace conditioning. Testing-induced ACh release during trace conditioning exceeded baseline levels in both brain regions, whereas testing-induced ACh levels during delay conditioning were not significantly greater than baseline levels. Interestingly, this pattern was not present in animals that did not adequately learn both tasks, which instead demonstrated no differences in ACh efflux as a function of task or brain region. Collectively, findings from this experiment demonstrate a continued involvement of cholinergic modulation in mPFC during performance of a previously acquired trace conditioning task, where cholinergic activity in the mPFC exceeds that observed in the HPC during trace conditioning.
and exceeds the level of cholinergic activity observed in either the mPFC or HPC during delay conditioning.

The finding that ACh levels were greater in the mPFC during performance in trace conditioning than during delay conditioning suggests that cholinergic modulation of the mPFC plays a key role in the performance of trace conditioning. This suggestion is consistent with previous findings that the mPFC contributes to both acquisition (Takehara-Nishiuchi et al., 2005) and performance (Simon et al. 2005; Takehara-Nishiuchi et al., 2006) in trace conditioning. For example, post acquisition lesions of the prelimbic cortex in the mPFC impair subsequent performance in trace conditioning (Simon et al., 2005), implying that this region of the mPFC plays a continuing role in the successful performance of the task.

The current finding of increased cholinergic activity in the mPFC during trace conditioning may be due a number of factors including the regulation of attention; trace conditioning, in contrast to delay conditioning, depends on attention (Han et al., 2003). Attention may play a part in maintaining a mental representation of the CS during the TI, where this maintenance might critically depend on cholinergic modulation of the mPFC. This argument is consistent with studies showing that lesions of the mPFC
retard, performance and increase preservative responding in attention demanding tasks (Chudasama & Muir, 2001; Dalley et al., 2004; Passetti, Chudasama, & Robbins, 2002). For example, Dalley and colleagues (2004) found that post-acquisition selective cholinergic lesions of the mPFC impair performance in a five-choice visual attention paradigm. They also found that during high attentional demands, subjects with cholinergic mPFC lesions demonstrated perseveration and increased anticipatory responding compared to control groups.

In the current task subjects demonstrated suppression of non-adaptive responding during the CS in the trace conditioning paradigm (see Figure 3). The findings of increased anticipatory responding and perseveration following cholinergic mPFC lesions as reported by others (Dalley et al., 2004), suggest that the mPFC may also play a role in suppressing non-adaptive responding during the CS interval in the trace conditioning task. This position is consistent with the finding that during trace eyeblink conditioning using whisker stimulation as the CS, whisker barrel lesions reduced adaptive responding during the TI and increased non-adaptive responding during the trace CS interval (Galvez et al., 2007).
ACh levels in the mPFC and HPC did not show an increase during delay conditioning, in agreement with previous literature showing that these regions are not necessary for the successful performance of this task (McLaughlin et al., 2002; Weiss, Bouwmeester, Power & Disterhoft, 1999). The contrast in the pattern of ACh release in mPFC and HPC during trace and delay conditioning observed in the current experiment provides further evidence that performance in these two classes of associative learning relies upon differing brain circuitry, and therefore represents truly different forms of memory.

While previous lesion studies have shown that the HPC is not necessary for post-consolidation performance of trace (or delay) conditioning, results from the current experiment show that ACh levels in the HPC were greater during trace conditioning than during delay conditioning and that testing-induced ACh release during trace conditioning exceeded basal levels of release. The finding of increased cholinergic modulation in the HPC during trace conditioning over that seen in delay conditioning is consistent with findings that the HPC plays a vital role in the acquisition (Bangasser et al.,
2006; Fendt et al., 2005; Misane et al. 2005; Takehara-Nishiuchi et al., 2005; Takehara-Nishiuchi et al., 2006; Yoon & Otto, 2007) and consolidation (Kim et al., 1995; Quinn et al., 2002) of trace, but not delay, conditioning.

It is possible that subjects in the present study had not yet fully consolidated the trace conditioning task, perhaps due to the increased difficulty of learning both the trace and delay conditioning tasks concurrently. It is also possible that the HPC plays a continuing role in timing the CR in trace conditioning (see Rodriguez & Levy, 2001), and thus cholinergic modulation of HPC may persist beyond initial acquisition of the trace conditioning response. Data suggesting HPC involvement in response timing in trace conditioning comes from studies of knock-out mice lacking an NMDA receptor subunit of the CA3 pyramidal cells (Kishimoto et al., 2006). Although these mice were able to demonstrate some acquisition of trace conditioning, they were unable to properly time their CRs during the training phase. Kishimoto and colleagues (2006) believe that adaptively timed CR acquisition requires recruitment of both CA1 and CA3 memory networks. With the CA3 network missing, animals
are still able to rely on CAI networks to activate the memory trace sufficiently to allow acquisition of the task, but without the precision that a fully intact hippocampal network would allow.

The finding that ACh efflux was not elevated in the HPC during delay conditioning is consistent with numerous studies demonstrating that lesions of the HPC disrupt acquisition of trace conditioning but spare acquisition of delay conditioning (Bangasser et al., 2006; Fanselow et al., 2005; Misane et al. 2005; Yoon & Otto, 2007). The finding that the HPC is not necessary to learn in delay conditioning paradigms has also been corroborated using both pharmacological manipulations and genetic knockout mice (Wanisch et al., 2004; Kishimoto et al., 2006).

In conclusion, results from the present study suggest that during trace, but not delay conditioning, cholinergic activation in the mPFC and HPC is important for post acquisition performance. This implicates the mPFC as a potential site for a sustained mental representation of the CS during the TI and/or suppression of non-adaptive responding. The HPC likely aids in the continued consolidation of the trace conditioning task, and may contribute to adaptive timing of the CR. The current findings contribute to our understanding of the
neurobiological substrates underlying trace and delay conditioning by delineating the pattern of cholinergic modulation of the mPFC and HPC, two brain structures known to play a selective role in trace and not delay conditioning.
REFERENCES


NMDA receptors within CA1 pyramidal neurons.

Neuron. 25, 473-480.


Takehara-Nishiuchi, K., Kawahara, S., & Kirino, Y. (2005). NMDA receptor-dependent processes in the medial PFC are important for acquisition and the early stage of consolidation during trace, but not delay eyeblink conditioning. Learning and Memory, 12, 606-614.


