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MAPPING LEARNING NETWORKS BY EXAMINING NEURONAL AND POPULATION ACTIVITY DURING TRACE CLASSICAL FEAR CONDITIONING

A Thesis in

Integrative Biosciences

by

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ABSTRACT

Identifying the neural substrates of learning and memory is an important topic in neuroscience research. A powerful approach to examining the neural basis of memory is electrophysiological recording of neural activity during the learning process. The experiments in this dissertation use single-site and multi-site electrophysiological recordings to examine neuronal activity during trace fear classical conditioning. Trace fear conditioning is a hippocampus-dependent task that requires a subject to associate a neutral conditioned stimulus (CS) and an aversive unconditioned stimulus (US) that are separated by several seconds. The first two studies in this dissertation examine single neuron activity in two important learning structures during trace fear conditioning. These studies show that neurons in the hippocampus and medial prefrontal cortex (mPFC) exhibit patterns of activity specific to learning and that the activity in each site may serve distinct but complementary roles in the acquisition of trace fear conditioning. The complementary activity in these sites suggests that these structures interact to encode a memory. Chapter 4 examines the field activity of populations of neurons simultaneously in the hippocampus and mPFC during trace fear conditioning. Chapter 4 also examines activity in the amygdala, a third important site in this learning network. These multi-site recordings show that the hippocampus and mPFC exhibit coordinated activity throughout training and that the hippocampus and amygdala exhibit coordinated activity late in training. Using various electrophysiological recording approaches, this dissertation suggests that the hippocampus, mPFC, and amygdala coordinate during different phases of the learning process to encode the CS-US association.
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Chapter 1

Introduction

1.1 Overview

Identifying the neural substrates of learning and memory is an important topic in neuroscience research. A powerful approach to examining the neural basis of memory is electrophysiological recording of neural activity during the learning process. The experiments in this dissertation use single-site and multi-site electrophysiological recordings to examine neuronal activity during trace fear classical conditioning. Trace fear conditioning is a hippocampus-dependent task that requires a subject to associate a neutral conditioned stimulus (CS) and an aversive unconditioned stimulus (US) that are separated by a trace interval of several seconds. The first two studies in this dissertation examine single neuron activity in two important learning structures during trace fear conditioning. These studies show that neurons in the hippocampus and medial prefrontal cortex (mPFC) exhibit patterns of activity specific to learning and that the activity in each site may serve distinct but complementary roles in the acquisition of trace fear conditioning. The complementary activity in these sites suggests that these structures interact to encode a memory. Chapter 4 examines the field activity of populations of neurons simultaneously in the hippocampus and mPFC during trace fear conditioning. Chapter 4 also examines activity in the amygdala, a third important site in this learning network. These multi-site recordings show that the hippocampus and mPFC exhibit
coordinated activity throughout training and that the hippocampus and amygdala exhibit coordinated activity late in training. Using various electrophysiological recording approaches, this dissertation suggests that the hippocampus, mPFC, and amygdala coordinate during different phases of the learning process to encode the CS-trace-US association.

1.2 Classical fear conditioning

Classical, or Pavlovian, fear conditioning is a form of learning that is quickly acquired by a subject. In classical fear conditioning, a neutral conditioned stimulus (CS) such as a tone is followed by an aversive unconditioned stimulus (US), such as an electric shock. The aversive US elicits multiple behavioral unconditioned responses from the subject, such as changes in heart rate (HR) and blood pressure (Iwata & LeDoux, 1988; Marchand & Kamper, 2000; McEchron, Cheng, & Gilmartin, 2004; McEchron, Tseng, & Disterhoft, 2000), cessation of movement (Blanchard & Blanchard, 1969; Fanselow, 1980), analgesia (Fanselow & Bolles, 1979; Fanselow & Helmstetter, 1988), and in humans, changes in galvanic skin conductance (Cheng, Knight, Smith, Stein, & Helmstetter, 2003; Knight, Nguyen, & Bandettini, 2003; Orr et al., 2000; Prokasy & Kumpfer, 1973). After repeated pairings of the CS and US, the previously neutral CS elicits similar behavioral responses indicative of fear (conditioned responses) in the subject. The studies in this dissertation use HR fear conditioning because conditioned changes in HR provide a reliable and continuous measure of learned fear (Marchand, 2002; Marchand & Kamper, 2000; McEchron, Alexander, & Gilmartin, *in preparation*).
The simplest form of classical fear conditioning is known as delay fear conditioning. In delay conditioning the presentation of the CS and US overlap. This dissertation examines the neuronal activity underlying a more complex form of classical fear conditioning, called trace fear conditioning. Trace fear conditioning requires subjects to associate the CS and US when they are separated by an empty 20-second trace interval. Trace fear conditioning requires additional higher-order learning structures (e.g., the hippocampus and mPFC), and this dissertation seeks to determine how activity in multiple structures contributes to trace fear acquisition. The experiments in this dissertation compare neuronal activity during trace fear conditioning to an unpaired pseudoconditioning control group. In the unpaired situation, the CS and US are presented to the subject on separate trials in a near-random order so that the CS never predicts the US (Rescorla, 1967). Neural and behavioral responses to the unpaired CSs and USs represent the level of no learning (i.e. sensitized responding to stimuli in an aversive context). Unpaired pseudoconditioning control training is a common control procedure in fear conditioning experiments (e.g., Maren, 2000; Maren, Yap, & Goosens, 2001; McEchron, Bouwmeester, Tseng, Weiss, & Disterhoft, 1998; McEchron, Cheng, & Gilmartin, 2004; Rogan, Staubli, & LeDoux, 1997).

1.3 Trace conditioning and the hippocampus

Trace fear conditioning requires an intact hippocampus. A number of studies have shown that pre- or post-training lesions of the hippocampus impair animals’ ability to associate the CS and US when these stimuli are separated by the trace interval, but not
in a delay situation where the CS and US overlap (Chowdhury, Quinn, & Fanselow, 2005; McEchron, Bouwmeester, Tseng, Weiss, & Disterhoft, 1998; McEchron, Tseng, & Disterhoft, 2000; Phillips & LeDoux, 1992; Quinn, Oommen, Morrison, & Fanselow, 2002). Similar results have also been found in another trace conditioning task, called trace eyeblink conditioning, in which an auditory-CS and an eyeblink-eliciting US are separated by a 500-ms trace interval. Trace eyeblink and trace fear conditioning share common neural pathways, and hippocampal lesions similarly impair the acquisition and retention of trace, but not delay eyeblink conditioning (Kim, Clark, & Thompson, 1995; Moyer, Deyo, & Disterhoft, 1990; Solomon, Vander Schaaf, Thompson, & Weisz, 1986; Takehara, Kawahara, Takatsuki, & Kirino, 2002; Tseng, Guan, Disterhoft, & Weiss, 2004; Weiss, Bouwmeester, Power, & Disterhoft, 1999).

The number of trace fear conditioning studies has grown rapidly in the past several years. When the experiments in this dissertation were first started, only a few trace fear conditioning studies existed in the literature. Now, because of its hippocampal dependence and ease of use in behavioral experiments, trace fear conditioning is used in many studies of hippocampal function. Thus, there is considerable interest in identifying the brain structures and neuronal activity patterns that govern trace fear conditioning. The usefulness of the trace fear conditioning model can be seen in studies examining the role of N-methyl-D-aspartate (NMDA) receptors (Huerta, Sun, Wilson, & Tonegawa, 2000; Misane et al., 2005; Quinn, Loya, Ma, & Fanselow, 2005) gamma-aminobutyric acid type A (GABA_A)-mediated transmission (Crestani et al., 2002; Wiltgen, Sanders, Ferguson, Homanics, & Fanselow, 2005), and cholinergic inputs in hippocampal processing (Hunt & Richardson, 2007; Moye & Rudy, 1987). A number of other studies
have used trace fear conditioning to examine the effects of nutrition during development and the effects of aging on hippocampal function (McEchron, Cheng, & Gilmartin, 2004; McEchron, Cheng, Liu, Connor, & Gilmartin, 2005; Moyer & Brown, 2006; Villarreal, Dykes, & Barea-Rodriguez, 2004). Trace fear conditioning and other hippocampus-dependent tasks have been used to examine the relationship between learning and adult neurogenesis in the dentate gyrus (Cuppini et al., 2006; Shors, Townsend, Zhao, Kozorovitskiy, & Gould, 2002). Given the growing use of trace fear conditioning in studies of hippocampal processing, it is important to identify the network of structures and neuronal activity responsible for trace fear learning. This dissertation examines the neuronal activity in the three major structures of the trace fear conditioning network: the hippocampus, medial prefrontal cortex, and amygdala.

1.4 Trace conditioning and extra-hippocampal structures

Numerous studies have established the importance of the hippocampus for the acquisition and retention of trace fear conditioned memories. Recently, several studies have identified the medial prefrontal cortex (mPFC) as an important part of the trace fear conditioning network. Chapter 3 sought to determine if neuronal activity in the mPFC is specific to the acquisition of trace fear memories. Findings from several trace conditioning paradigms have contributed to our interest in this structure. Damage to the mPFC impairs the acquisition and retention of trace eyeblink conditioning (Kronforst-Collins & Disterhoft, 1998; McLaughlin, Skaggs, Churchwell, & Powell, 2002; Powell, Churchwell, & Burriss, 2005; Powell, Skaggs, Churchwell, & McLaughlin, 2001; Simon,
Knuckley, Churchwell, & Powell, 2005; Takehara, Kawahara, & Kirino, 2003; Weible, McEchron, & Disterhoft, 2000), and disruption of kinase activity in the mPFC impairs the consolidation of trace fear memories (Runyan & Dash, 2004; Runyan, Moore, & Dash, 2004; Villarreal & Barea-Rodriguez, 2006).

According to Hebbian theories of trace classical conditioning, some brain structure(s) must provide a bridging mechanism during the trace interval so that a representation of the CS can overlap with the US (e.g., Levy, Sanyal, Rodriguez, Sullivan, & Wu, 2005; Mongillo, Amit, & Brunel, 2003; Reutimann, Yakovlev, Fusi, & Senn, 2004; Rodriguez & Levy, 2001; Wallenstein, Eichenbaum, & Hasselmo, 1998). Several lines of evidence suggest that the role of the mPFC during trace fear conditioning may be to bridge the temporal gap separating the CS and US. Studies in primates using delayed-response tasks have demonstrated the importance of the mPFC for learning tasks that require stimulus information to be held in memory across a delay period (Funahashi, Bruce, & Goldman-Rakic, 1993; Fuster, 1989). Other work shows that single neurons in the mPFC of rats and primates exhibit tonic firing responses during empty intervals separating stimuli and behavioral responses (J. Y. Chang, Chen, Luo, Shi, & Woodward, 2002; Fuster, 1973, 1990). Functional magnetic resonance imaging during human trace fear conditioning has indicated that frontal cortical regions are activated during the trace interval separating the CS and US (Knight, Smith, Cheng, Stein, & Helmstetter, 2004).

Chapter 3 of this dissertation sought to determine whether single neurons in the mPFC exhibit sustained increases in activity during the trace interval that may serve to bridge the gap between the CS and US.
The final study in this dissertation examines the coordination of neuronal activity in the primary learning and memory structures of the trace fear conditioning network (hippocampus, mPFC, and amygdala). Along with the hippocampus and mPFC, the amygdala is hypothesized to be a critical structure for trace fear conditioning. These three structures show functional anatomical connections (Chiba, 2000; Chiba, Kayahara, & Nakano, 2001; Ishikawa & Nakamura, 2003, 2006; Jay & Witter, 1991; Likhtik, Pelletier, Paz, & Pare, 2005; Pitkanen, Pikkarainen, Nurminen, & Ylinen, 2000; Quirk, Likhtik, Pelletier, & Pare, 2003). The amygdala is critical for the acquisition and permanent storage of delay fear conditioning (Collins & Pare, 2000; Fanselow & LeDoux, 1999; Gale et al., 2004; LeDoux, Cicchetti, Xagoraris, & Romanski, 1990; Pare, Quirk, & Ledoux, 2004; Phillips & LeDoux, 1992; Repa et al., 2001; Romanski, Clugnet, Bordi, & LeDoux, 1993; Schafe, Doyere, & LeDoux, 2005; Walker & Davis, 2000). The amygdala is also necessary for the hippocampus-dependent contextual fear conditioning task, in which the contextual cues of the training environment are associated with the US (Cousens & Otto, 1998; Gale et al., 2004; Goosens & Maren, 2001; Maren, Aharonov, Stote, & Fanselow, 1996; Phillips & LeDoux, 1992). Clearly, the amygdala is involved in most, if not all, forms of fear learning. Chapter 4 will examine the field activity in the amygdala in addition to the hippocampus and mPFC to determine whether it interacts with the other sites in this network to encode information during trace fear conditioning.
1.5 Single neuron activity in trace conditioning

Prior to this dissertation work, relatively little was known about the neuronal activity underlying trace fear conditioning. As a result, some of the background for the present studies comes from single neuron and multiunit recordings during trace eyeblink conditioning in rats and rabbits. Neurons recorded from the CA1 area of the hippocampus showed learning-related increases in firing during the tone-CS that precede the emergence of conditioned eyeblink responses (McEchron & Disterhoft, 1997; McEchron, Weible, & Disterhoft, 2001; Solomon, Vander Schaaf, Thompson, & Weisz, 1986; Weible, O'Reilly, Weiss, & Disterhoft, 2006; Weiss, Kronforst-Collins, & Disterhoft, 1996; Woodruff-Pak, Lavond, Logan, & Thompson, 1987). The Disterhoft laboratory also examined prefrontal activity during trace eyeblink conditioning. Single neurons recorded in the anterior cingulate cortex, a site that is adjacent to the mPFC, exhibited increases in activity during the CS and trace interval; this pattern persisted over subsequent days of conditioning in the paired group, but not in the unpaired group (Weible, Weiss, & Disterhoft, 2003). An important difference between trace eyeblink and trace fear conditioning is that the CS-US association in trace fear conditioning can be made across a long trace interval with a duration of 10-30 s. In contrast, the trace eyeblink association cannot be made if the trace interval is longer than 1 s. Because of the short trace interval in trace eyeblink conditioning, it is difficult to clearly distinguish neuronal activity that is specific to the trace interval and independent of CS-induced changes in activity. The experiments in this dissertation examine neuronal activity
during the longer trace interval in trace fear conditioning to determine whether single neurons exhibit activity that provides a bridging signal between the CS and US.

An earlier study that preceded this dissertation work examined single neuron activity in the CA1 area of the rabbit hippocampus during trace fear conditioning (McEchron, Tseng, & Disterhoft, 2003). This study showed that CA1 single neurons exhibit phasic increases in activity that encode information about the duration of the trace interval. The experiment in Chapter 2 of this dissertation examines hippocampus single neuron activity during trace fear conditioning in the rat to determine whether single neuron activity in the hippocampus encodes learning-related information about the CS, US, and trace interval during acquisition. Furthermore, the experiment in Chapter 2 examines the dentate gyrus (DG) and CA1 regions to determine whether the first and last site in the tri-synaptic loop of the hippocampus show different patterns of encoding during trace fear conditioning.

The experiment in Chapter 3 examines single neuron activity in two regions of the mPFC during trace fear conditioning to determine whether this structure exhibits learning-related patterns of activity specific to the CS and the long 20-s trace interval. Single neuron activity has been examined previously in the mPFC during trace fear conditioning using a short 2-s trace interval (Baeg et al., 2001). This study showed that mPFC neurons exhibit some level of sustained firing during the trace interval (Baeg et al., 2001). The experiment in Chapter 3 shows that a subset of mPFC neurons exhibit sustained firing during a longer 20-s trace interval.
1.6 Neuronal oscillations in learning and memory

Field activity or electroencephalographic (EEG) recording is another electrophysiological approach that has allowed researchers to understand how a brain region encodes information during learning. Field activity represents the combined activity of a population of neurons (Mitzdorf, 1985). The experiment in Chapter 4 of this dissertation examines field activity within the three major sites in the trace fear learning network (i.e., the hippocampus, mPFC, and amygdala) to determine if rhythmic population activity is coordinated between multiple sites. Rhythmic, or oscillatory, activity is a prominent feature of EEG signals that occurs when a population of neurons fire together in a rhythmic pattern. The most studied rhythm in the mammalian brain with regard to learning and memory is the theta rhythm. Theta is a 3-8 Hz oscillation that is most prominent in the hippocampus (Kramis, Vanderwolf, & Bland, 1975; Vanderwolf, 1969). Several studies have shown that this rhythm is related to learning and remembering various forms of information (Berry & Thompson, 1978; B. S. Givens & Olton, 1990; Jacobs, Hwang, Curran, & Kahana, 2006; Raghavachari et al., 2001). In eyeblink conditioning, the amount of hippocampal theta during a training session is correlated with the rate of acquisition of conditioned responses (Berry & Thompson, 1978). Furthermore, if paired trials are delivered only when the theta rhythm is present, rabbits acquire the conditioned eyeblink response faster than if trials are delivered only in the absence of theta (Griffin, Asaka, Darling, & Berry, 2004; Seager, Johnson, Chabot, Asaka, & Berry, 2002).
Some evidence suggests that coordination of theta rhythmic activity across multiple structures may be important for fear learning. A recent study examined the coordination of rhythmic activity in the hippocampus and amygdala during a differential fear conditioning paradigm (Pape, Narayanan, Smid, Stork, & Seidenbecher, 2005; Seidenbecher, Laxmi, Stork, & Pape, 2003). This study showed that field activity in the hippocampus and amygdala became correlated at a frequency in the theta range during the retention, but not acquisition, of conditioned fear memories (Pape, Narayanan, Smid, Stork, & Seidenbecher, 2005; Seidenbecher, Laxmi, Stork, & Pape, 2003). Chapter 4 of this dissertation examines neuronal oscillations in the mPFC, hippocampus, and amygdala to determine whether rhythmic activity in these structures becomes coordinated during the CS, US, or trace interval in trace fear conditioning.

1.7 Summary of rationale

In summary, the experiments included in this thesis sought to determine whether individual neurons and populations of neurons in three major learning and memory structures exhibit patterns of activity specific to the acquisition of a trace fear classical conditioning task. First, because trace fear conditioning is a hippocampus-dependent task, we sought to determine whether single neurons in the CA1 and DG region of the hippocampus exhibit learning-related changes in activity to the CS, US, and trace interval during trace fear conditioning (Chapter 2). Second, because the association of the CS and US in trace fear conditioning requires maintaining stimulus information across an empty trace interval, we sought to determine whether activity in the mPFC exhibits tonic
increases during the trace interval that could bridge the temporal gap between the CS and US (Chapter 3). Finally, we examined the rhythmic field activity of neuronal populations in the hippocampus, mPFC, and amygdala simultaneously to determine whether these structures exhibit coordinated activity during various phases of trace fear conditioning (Chapter 4).
Chapter 2
Single Neurons in the Dentate Gyrus and CA1 of the Hippocampus Exhibit Inverse Patterns of Encoding during Trace Fear Conditioning

2.1 Introduction

The experiment in this chapter examined hippocampal single neuron activity during trace fear conditioning to determine if the hippocampus encodes information about the CS and US, as well as provide patterns of activity that could bridge the trace interval. This study was published in 2005 (Gilmartin & McEchron, 2005a). Trace fear conditioning is a learning task that requires animals to associate a neutral CS and a fear-producing shock-US that are separated by a silent 20-second trace interval. The acquisition and retention of trace fear conditioning, similar to other forms of trace conditioning, requires an intact hippocampus (Chowdhury, Quinn, & Fanselow, 2005; Kim, Clark, & Thompson, 1995; McEchron, Bouwmeester, Tseng, Weiss, & Disterhoft, 1998; McEchron, Tseng, & Disterhoft, 2000; Moyer, Deyo, & Disterhoft, 1990; Quinn, Oommen, Morrison, & Fanselow, 2002; Solomon, Vander Schaaf, Thompson, & Weisz, 1986). A delay fear conditioning paradigm, in which there is no trace interval separating the CS and US, does not require the hippocampus (Chowdhury, Quinn, & Fanselow, 2005; McEchron, Bouwmeester, Tseng, Weiss, & Disterhoft, 1998; McEchron, Tseng, & Disterhoft, 2000; Phillips & LeDoux, 1994; Quinn, Oommen, Morrison, & Fanselow, 2002). The critical role of the hippocampus for trace, but not delay, conditioning tasks
suggests that single neurons in this structure may serve to bridge the trace interval. Previous recording studies using trace eyeblink conditioning have shown that CA1 neurons encode information about the CS and US and exhibit some increases during the trace interval (McEchron & Disterhoft, 1997; McEchron, Weible, & Disterhoft, 2001; Solomon, Vander Schaaf, Thompson, & Weisz, 1986).

Recently, McEchron and colleagues recorded single neuron activity from the CA1 area of the rabbit hippocampus during trace fear conditioning with a 10- or 20-second trace interval (McEchron, Tseng, & Disterhoft, 2003). This study showed that CA1 single neurons exhibit learning-related increases in activity on CS-alone trials timed to the duration of the trace interval; however, patterns of activity to the CS, US, and trace interval showed only moderate changes. The present study expanded on the findings of this recent study by recording single neurons simultaneously in the DG and CA1 hippocampal areas of rats during trace fear conditioning. This allowed us to determine how the first and last synaptic regions within the tri-synaptic loop of the hippocampus (i.e., the DG and CA1) encode learning-related information about the CS, US and trace interval during trace fear conditioning. Specifically, we hypothesized that hippocampal single neurons encode information about the CS and US and exhibit patterns of activity during the trace interval that could bridge the CS-US gap. A within-subjects learning control was implemented whereby rats first received non-associative unpaired pseudoconditioning control training followed by trace fear conditioning. This design minimized between-groups variability in single neuron response patterns which could be attributed to different electrode placements within the hippocampus. Moreover, it
allowed for a comparison of single neuron activity on an individual fixed electrode between an associative and a non-associative situation.

2.2 Materials and Methods

2.2.1 Subjects and surgery

Surgery was performed on a total of 18 Brown Norway/F344 female rats (200-400 g; Harlan, IN) in this study. The majority of the analyses in this chapter were performed on 6 of the rats that yielded reliable single neuron activity from both the DG and CA1 throughout the pseudoconditioning sessions and the subsequent trace fear conditioning sessions (i.e., a total of 9 consecutive days of training). Another 8 rats were used for analyses that verified the overall pattern of DG and CA1 single neuron responses to the CS and US. These 8 rats received only 2 days of habituation/acclimation followed by 2 days of trace fear conditioning. The remaining 4 rats received electrode surgeries but were not included in any analyses in this study because of improper electrode placement or unstable recordings during training. All rats were housed individually and received food and water *ad libitum*. All surgical procedures were in accordance with the National Institutes of Health and the Pennsylvania State University Institutional Animal Care and Use Committee. Rats were anesthetized with 1-2% halothane in 100% O₂ during surgery. Eyes were kept moist with antibacterial ophthalmic ointment. Each rat was positioned in a stereotaxic frame and the skull was exposed. Five self-tapping screws (#0 4.75mm, Small Parts, Inc., FL) were inserted ~1 mm into the skull to anchor
the final dental cement head assembly. A 3-mm diameter hole was drilled in the skull above the left dorsal hippocampus, and an 8-electrode array was stereotaxically lowered into the dorsal hippocampus. The array consisted of eight Teflon-coated stainless steel electrodes arranged in two rows of four electrodes (50 µm diameter each; tip separation: 200-400 µm; NB Labs, Dennison, TX). One row was cut 1.20 mm longer than the other. This array configuration resulted in the short row of electrodes being positioned in the CA1 pyramidal cell layer and the longer row in the dentate gyrus (DG) granule cell layer. As the electrode-array was lowered into the brain the caudal-most electrodes were oriented approximately 0.5 mm more laterally and 0.5 mm more ventrally compared with the rostral-most electrodes in order to target the DG granule cell layer which changes its orientation rostrocaudally. Single neuron activity was monitored from two DG and two CA1 channels while the electrodes were being lowered to enable optimal electrode placement. Dental cement was then used to secure the electrodes to the skull and close the remaining wound area. Skull screws were used as the ground and reference electrode.

2.2.2 Heart rate fear conditioning

Prior to training, 6 of the rats were acclimated to the restrainer and conditioning chamber for four days (20 minutes/day) to reduce fear or arousal to handling or restraint. No stimuli were presented during the acclimation sessions. During acclimation, training, and testing sessions, rats were restrained in a flexible cotton bag and placed in an 8-cm wide U-shaped Plexiglas restrainer located within a sound-attenuating chamber. The restrainer allowed for some movement, but prevented the rat from turning around.
Electrocardiographic (EKG) activity was recorded across two leads placed on the chest or across leads placed at the neck and lower back. The EKG leads were either stainless steel wound clips or mesh wires pressed to the skin with conductive gel. The EKG activity was amplified 10,000X, filtered between 10 and 1000 Hz, and sampled at 25 kHz. The ends of copper tubes (5 mm diameter) were placed 1 cm from the opening of each ear and served to deliver the auditory tone conditioned stimulus (CS; 5 sec; 6000 Hz; 80 dB). Two flexible wire mesh leads were secured to the rat’s tail to deliver the unconditioned shock stimulus (US; 0.8 sec; 0.75-mA alternating current).

One day after the fourth acclimation session, each of the 6 rats received three consecutive days of unpaired pseudoconditioning control training, followed 24 hours later by a session of CS-alone trials. Forty-eight hours following this CS-alone session, the same rats received three consecutive days of trace fear conditioning. Rats then received one session of CS-alone retention trials 24 hours later.

Unpaired pseudoconditioning. The pseudoconditioning sessions were used to determine the non-associative level of behavioral and neuronal responding. Each pseudoconditioning session consisted of 6-10 CS-alone trials and 6-10 US-alone trials using an intertrial interval (ITI) of 100 ± 20 sec. For several animals restraint stress caused struggling after 6 CS-alone and 6 US-alone trials on several of the training days. For these cases training was halted on this day, and additional trials were delivered on the following day of training. During each pseudoconditioning session, the same stimulus was never presented more than two consecutive times. One day after the last pseudoconditioning session, rats received one US-alone reminder trial followed by 6-10
CS-alone trials. Two of the six rats received two additional US-alone trials interspersed among the CS-alone retention trials.

*Trace fear conditioning.* Forty-eight hours following the pseudoconditioning-CS-alone session, each rat received three consecutive days of 20-second trace fear conditioning. Each 20-second trace fear conditioning session consisted of 6-10 paired CS-US trials (ITI = 200 ± 20 sec), in which the offset of the tone-CS was separated from the onset of the shock-US by an empty 20-second trace interval on each trial. Twenty-four hours after the third trace fear conditioning session, rats received 6-10 CS-alone retention trials. Each rat received 1 or 2 paired CS-trace-US trials interspersed among the CS-alone retention trials.

2.2.3 Single neuron recording

Single neuron analog signals were amplified (10,000X), filtered (300 Hz to 5 kHz), and collected with a DT-3100 Data Translation board (Data Translation, Marlboro, MA) attached to an 800-MHz computer, which sampled each channel at 25 kHz. The same computer sampled EKG activity and controlled the delivery of all stimuli. Single neuron and EKG data were collected from 16 seconds before CS onset to 16 seconds after US onset using software developed in our laboratory.

Single neurons were extracted from the continuous data stream using an offline spike height threshold detection software routine. This routine extracted 1.4-ms epochs of data with a significant (p<0.05) rise and fall that occurred within a 1.0-ms window. A second software routine used template-matching algorithms to separate all individual
single neuron waveforms on an electrode. These spike extraction and separation programs are based on software techniques developed by Dr. Matthew McEchron and Dr. John Disterhoft at Northwestern University. These methods and software routines were developed specifically for the purpose of eliminating noise and artifact signals from the data record, and separating individual neurons recorded from an electrode with a high level of reliability. These spike cutting routines were originally validated by comparing them to the *DataWave Cluster Cutting* software package (Longmont, CO), and they were used and described in McEchron et al. (McEchron, Tseng, & Disterhoft, 2003; McEchron, Weible, & Disterhoft, 2001). The McEchron and Disterhoft software routines were later re-written in the McEchron laboratory using a faster user-friendly 32-bit Visual Basic 5.0 platform (*Microsoft Corp*; Seattle, WA). The spike separation software allowed for multiple template windows to be defined for the characteristic waveform of each single neuron. An infinite number of window limits could be defined along any pixel-point and at any angle along the characteristic spike waveform. All action potential waveforms that fell within the multiple window boundaries of a single spike template were clustered as an individual single neuron. Voltages from the peak and subsequent trough of each spike were plotted to insure that spike-clusters corresponded to individual neurons, and to insure that all spike clusters were greater than the basal level of non-spike noise. In many cases spikes were also clustered according to spike width and the spike angle between peak and trough. This clustering approach has been described in other studies (e.g., Gray, Maldonado, Wilson, & McNaughton, 1995; B. L. McNaughton, O'Keefe, & Barnes, 1983) and is now used in other commercially available software packages (e.g., *DataWave Technologies*; Longmont, CO). As a general rule, spikes had
to be at least twice as great as the basal peak level of noise. On average, however, spikes were 4.5 times greater than the basal noise level. All action potential waveforms recorded from an electrode were compared visually to ensure that the characteristic waveform of each single neuron cluster was different from the characteristic waveforms of all other clusters. This conservative approach ensured that the ensembles recorded from each electrode were made up of unique single neurons that could be accurately followed throughout a single training session. It also ensured that no noise or artifact signals were included in the final spike data. Any artifact or aberrant waveform could easily be eliminated by including another template window along the waveform. The software also included a millisecond timestamp analysis that allowed the user to determine if there was a temporal relationship between smaller spike heights that followed larger spikes within a window of 15 ms. Smaller spikes that consistently followed larger spikes within this time window were reanalyzed as a single spike cluster corresponding to a complex spike event. It is important to note that the configuration of single neurons on an electrode changed from one day of recording to the next in almost all cases. For this reason, each day’s recorded single neurons were treated as a new set of neurons.

2.2.4 Analyses

All statistical analyses were performed with the aid of Microsoft (Redmond, WA) Visual Basic routines developed in our laboratory, Minitab Statistical Software version 10.0 (State College, PA), and Statistica software version 6.0 (Tulsa, OK). Single neuron
background firing rates were determined by calculating the mean discharge rate of each neuron during the 16-second baseline period before CS-onset on each trial. Changes in single neuron firing to the CS, trace interval, and US were measured on each trial using standard z-test change scores. The z-scores were calculated by subtracting the average baseline activity from the activity in a 5-second window during the trial. This difference in activity was then divided by the baseline variability. For units with very low baseline firing rates (i.e., < 0.10 Hz), the z-score was equivalent only to the difference score, and not divided by the baseline variability (standard deviation). This reduced the possibility that very low firing units would contribute inflated z-score values. For example, if a cell exhibited 1 spike during the 16 second baseline period on a trial (i.e., a baseline firing rate of 0.0625 Hz), and fired 2 spikes during the 5 sec CS, this would result in a corrected z-score of $z = 1.69$ (i.e., 2 spikes – average baseline firing of 0.31 spikes/5sec baseline). This z-score without the correction would be $z = 5.10$.

The z-scores were used to assess the magnitude of increases or decreases in firing during a specific time window during the trial. The z-scores could be averaged across trials or within a group of neurons. In most cases, these z-scores compared the activity in 5-second periods during the CS, trace interval, or post-US period to the mean activity during the 16-second baseline. Single neurons with z-scores greater than 3 standard deviations of the mean were capped at this level. Less than 3% of the standard score data were capped. Group comparisons of standard scores were performed using factorial repeated-measures ANOVAs that included the following factors: a repeated measure of 2-trial blocks within each day; a between factor of 4 days of training (i.e., 3 acquisition + 1 retention day); and a between factor of training condition (trace vs. unpaired). The
number of single neurons varied from one day to the next in each animal; however, the same single neurons were tracked accurately within each day. Thus, the trial-blocks factor was the only repeated measure used in these analyses. A Duncan’s multiple-range post-hoc test was used to test the significance of mean differences. Proportions of neurons were compared between groups using a non-parametric z-ratio-two-sample test of two independent proportions (described by Rosner, 1990). An $\alpha$ level of 0.05 was required for significance in all analyses.

Average histograms were constructed using normalized firing rates. For each single neuron, action potentials were summed across 4 trials into 1-second bins. Activity in each bin was then normalized to the mean and standard deviation of the basal firing rate. The normalized activity in each bin was averaged across groups of neurons to produce the histograms.

Software routines developed in our laboratory measured HR by calculating the time between the R-peaks in the raw EKG waveform and calculating beats per minute in each second. Changes in HR were analyzed using repeated measures ANOVAs, with an $\alpha$ level of 0.05 required for significance. All HR records containing arrhythmias, high baseline variability, or excessive movement artifact were excluded from analyses. These criteria resulted in four trials being excluded from the final analyses.

2.2.5 Histology

Marking lesions were placed at the tips of all electrodes by passing direct current (20 $\mu$A) for 15 seconds. Rats were deeply anesthetized with 5% halothane in 100% $O_2$. 
sacrificed by decapitation, and the brains were placed in a 10% formalin solution (0.9% saline). Brains were then frozen, sectioned coronally, mounted on glass slides, and stained with neutral red. A light microscope was used to locate electrode tips.

2.3 Results

2.3.1 Electrode placement and single neuron firing characteristics

The majority of the analyses were conducted on the 6 rats that showed reliable DG and CA1 recordings throughout unpaired pseudoconditioning and trace conditioning sessions. From these 6 rats, 18 electrodes were placed directly in the DG area and 14 were placed in the CA1 area. Of the electrodes placed in the DG and CA1 area of these 6 rats, 16 DG-electrodes and 13-CA1 electrodes yielded reliable single neuron data. Part A of Figure 2-1 shows an exemplar placement of electrodes in the left DG and CA1 from one of the 6 rats. All 6 rats had similar DG electrode placements; however, the caudal-most 1 or 2 CA1 electrodes were placed too dorsally in 5 of the 6 rats. Most DG electrodes were located in the lower blade of the DG. Electrodes placed outside of DG or CA1 were excluded from analyses.
Figure 2-1: Electrode placements and separated hippocampal single neurons.

A. Coronal diagrams show an exemplar placement of electrodes in the left DG and CA1 of the dorsal hippocampus from one rat used in this study. The X shows the placement of the most rostral and most caudal DG and CA1 electrodes. Similar DG placements were observed for all 6 rats. The caudal-most CA1 electrodes were placed too dorsally in 5 of the 6 rats. B. Approximately 1-7 units could be separated reliably on each electrode. Waveforms are shown for individual single neurons recorded from a characteristic DG or CA1 electrode from each of the 6 rats, and separated with template matching software. Single neuron waveforms are overlayed across 4 trials of activity. Bars = 0.1 mV and 0.5 msec. Coronal diagrams adapted from Paxinos and Watson (1998).
Approximately 1-7 single neurons were separated on each of the electrodes that yielded reliable data. During pseudoconditioning-control training an average of 65 DG units (range: 56-85) and 51 CA1 units (range: 39-74) were recorded per day of training. During subsequent trace conditioning an average of 67 DG units (range: 57-88) and 54 CA1 units (range: 45-69) were recorded on each day of training. Part B of Figure 2-1 shows waveforms for individual single neurons recorded from a characteristic DG or CA1 electrode from each of the 6 rats. Using template matching software these individual single neurons were separated reliably and free of artifact and noise. The baseline firing rate for each single unit was averaged across the first four trials of each day of training. The distributions of baseline firing rates for all recorded neurons revealed that a clustered majority of DG neurons (91%) had baseline firing rates below 5 Hz, and a large cluster of CA1 neurons (95%) had baseline firing rates below 8 Hz. Units in DG and CA1 firing faster than 5 Hz and 8 Hz, respectively, were considered interneurons. These units were excluded from analyses because the number of interneurons recorded was too small to provide statistically reliable results. These firing rate cut-offs are analogous to criteria described by Fox & Ranck (1975) and Nitz & McNaughton (1999, 2004). The mean baseline firing rate for all DG and CA1 neurons used in our analyses was 1.07 ± 0.05 Hz (S.E.M.) and 1.58 Hz ± 0.07 Hz (S.E.M.), respectively. ANOVAs conducted with Training (Trace vs. Pseudoconditioning) and Days (1, 2, 3, and Retention) factors revealed no significant effects for baseline firing in CA1; however, there was a significant Training effect for DG baseline firing, F(1,525) = 18.37; p = 0.00001. Although significant, baseline firing during unpaired pseudoconditioning was greater than during trace conditioning by only 0.3 Hz. This
difference may reflect the different pattern of stimulus presentations between the trace conditioning and pseudoconditioning situations. The mean spike width, or time from peak to trough, was 0.37 msec (±0.012 S.E.M.) for DG and 0.45 msec (±0.011 S.E.M.) for CA1 units recorded in this study.

2.3.2 Heart rate fear conditioning

Changes in HR were used to indicate learned fear responses. This has been shown to be a reliable measure of fear conditioning in the rat (Marchand, 2002; Marchand & Kamper, 2000; McEchron, Cheng, & Gilmartin, 2004). For all analyses, the change in heart rate was averaged into one-second bins for each animal across the 4th

![Figure 2-2: Conditioned heart rate (HR) fear responses.](image)

A. Each line depicts the change in HR from baseline in one-second bins averaged across CS-alone trials 4-7 on the retention day following trace or pseudoconditioning. Animals exhibited a larger increase in HR during the CS and a larger decrease in HR during the trace interval in trace compared with pseudoconditioning (Pseudo). Although no USs were delivered on these CS-alone trials, the open arrowhead marks the latency of US delivery on previous training trials. B. Each point represents the mean absolute change in HR averaged across the later trials of each day of training (trials 4-7). Absolute HR change was calculated by comparing the HR change during the tone-CS to the last 15 seconds of the trace interval period. Rats showed a greater absolute HR change during the later trials of trace conditioning compared with pseudoconditioning.
through the 7th CS trials of each day of training (i.e., CS-trace-US trials from trace conditioning and CS-alone trials from pseudoconditioning). These trials were used for all analyses because 3 animals showed movement artifacts during the initial trials of the initial days of pseudoconditioning. Moreover, trials 4-7 encompassed the final trials of each day in the majority of cases, and therefore comprised the maximal amount of learned fear. Figure 2-2A shows the mean change in HR from baseline averaged across CS-alone retention trials 4-7 following trace and unpaired pseudoconditioning. This figure shows that on CS-alone retention trials rats showed a larger increase in HR during the CS and a larger decrease in HR during the trace interval in the trace conditioning situation compared with the pseudoconditioning situation. Similar HR changes occurred on previous days of training in the trace situation, but these HR changes were largest on the retention day. Figure 2-2B shows the mean absolute HR change to the CS for each day of training. The absolute HR change was calculated by comparing the mean HR change during the 5-second tone-CS to the mean HR change during the last 15-seconds of the trace interval (the period starting 5-seconds after CS-offset). These parameters were selected a priori because our previous HR recordings have shown consistently that the HR response during trace fear conditioning trials is made up of an initial tachycardia which occurs during the 5-sec CS. This is typically followed by a 5-sec transition period between the initial tachycardia and the subsequent bradycardia (i.e., the first 5-sec of the trace interval), and the subsequent bradycardiac decrease in HR which consumes most of the last 15 seconds of the trace interval. Analyses conducted on the data in this graph showed that there was a greater absolute HR response to the CS during trace conditioning
compared with unpaired pseudoconditioning \[ \text{Training main effect: } F(1,5) = 48.34; p = 0.0009 \].

\section*{2.3.3 DG and CA1 population responses}

Figure 2-3 shows the average normalized peri-event time histograms for all DG neurons across all pseudoconditioning and trace conditioning sessions. These average histograms show that the population of DG single neurons exhibited an increase in activity to both the tone-CS and shock-US. There was a larger response to the tone-CS on days 2 and 3 in the trace conditioning situation compared with the pseudoconditioning situation. There was also an increase in activity to the US in both the trace and pseudoconditioning situation, but this increase was more sustained in the trace conditioning situation on days 2 and 3. Very little activation was seen during the trace interval in the trace and pseudoconditioning situations. Increases in DG responding to the CS and US can also be seen in the individual single neuron histograms in Figure 2-9.

Figure 2-4 shows the average normalized peri-event time histograms for all CA1 neurons across all pseudoconditioning and trace conditioning sessions. Single neurons recorded from CA1 during pseudoconditioning showed little response to the tone-CS, but CA1 neurons did show an excitatory response to the tone on day 1 of trace fear conditioning. Interestingly, CA1 exhibited a decrease in activity to the tone-CS on the retention day following trace conditioning. Decreases in CA1 responding to the CS can also be seen in the individual single neuron histograms in Figure 2-9. The CA1 neurons showed similar increases in activity following the US during the trace and
pseudoconditioning situations. The CA1 increases to the US were strongest early in training and diminished across days. No CA1 activation was seen during the trace interval period in either the trace or pseudoconditioning situation.

Figure 2-3: *Average peri-event time histograms for DG single neurons.*

Histograms show the mean normalized firing rate in one-second bins for all DG single neurons during the first four trials on each day of acquisition and on CS-alone retention trials. Firing rates in each bin were normalized to the mean baseline firing for each individual neuron. Filled arrowheads mark the time-point of the delivery of the shock-USs on 20-second trace conditioning trials (top panels) and on US-alone trials during pseudoconditioning control training (bottom panels). The CS-alone trials during pseudoconditioning are shown in the middle panels. Although no USs were delivered on CS-alone retention trials following trace fear conditioning, the open arrowhead marks the latency of US-delivery on previous training trials. The single neurons recorded from DG during trace conditioning showed greater increases in activity during the tone-CS and following the shock-US on Days 2 and 3 compared with the neurons recorded from DG during pseudoconditioning. Shock-US artifact resulted in little or no activity recorded during the US presentation.
Figure 2-4: Average peri-event time histograms for CA1 single neurons.

Histograms show the mean normalized firing rate in one-second bins for all CA1 single neurons during the first four trials on each day of acquisition and on CS-alone retention trials. Firing rates in each bin were normalized to the mean baseline firing for each individual neuron. Filled arrowheads mark the time-point of the delivery of the shock-USs on conditioning trials during 20-second trace fear conditioning (top panels) and on US-alone trials during pseudoconditioning (bottom panels). The CS-alone trials during pseudoconditioning are shown in the middle panels. Although no USs were delivered on CS-alone retention trials following trace fear conditioning, the open arrowhead marks the latency of US-delivery on previous training trials. The CA1 single neurons recorded from the trace conditioning group showed a small increase in activity during the tone-CS on day 1 which developed into a decrease in activity by the retention day. Shock-US artifact resulted in little or no activity recorded during the US presentation. This resulted in several panels showing no bar near the US.
2.3.4 DG and CA1 responding to the tone-CS

Analyses show that DG and CA1 single neurons show opposite patterns of responding to the tone-CS across trace fear conditioning. Moreover, the DG neurons showed on average a learning-related increase in activity to the tone-CS starting on Day 2, while CA1 neurons showed a learning-related decrease in activity later in training. These analyses were accomplished by calculating a z-score measure of the change in activity for each individual neuron during the tone-CS. These z-scores compared the firing activity during the 5-second tone-CS to the average activity in baseline. Figure 2-5A shows the mean change in single neuron activity in DG during the tone-CS in 2-trial blocks within each day. This figure shows that single neurons recorded from DG during the second and third days of trace conditioning exhibited greater increases in activity during the CS compared with neurons recorded during pseudoconditioning. A repeated measures ANOVA showed a significant Training X Day interaction, F(3,512) = 4.85; p = 0.0024. Follow-up tests revealed a significant difference between the Training conditions on Days 2 and 3.
Figure 2-5: Inverse DG and CA1 single neuron response patterns to the tone-CS.

Graphs show the mean change in single neuron activity from baseline during the 5-second tone-CS for units recorded from the DG (panel A) and the CA1 (panel B) areas of the dorsal hippocampus. Each point represents the mean standardized change in activity during the CS in a 2-trial block on each day of acquisition (Days 1-3) and on CS-alone retention trials (Ret). Error bars = S.E.M. Arrowhead on the ordinate axis shows the level of no change in activity.

Figure 2-5B shows the mean change in CA1 single neuron activity during the tone-CS in 2-trial blocks within each day of training. A small increase in CA1 responding was exhibited to the tone-CS during pseudoconditioning, which did not change over days. In contrast, there was a slightly larger increase in activity to the tone-CS on day 1 of trace fear conditioning. This was followed by a smaller response across subsequent days of trace conditioning which became a decrease in firing by the retention day. A repeated measures ANOVA showed a significant interaction of Training X Day, $F(3,403) = 3.46; p = 0.0199$. Follow-up tests revealed a significant difference between Training conditions on the retention day. Follow-ups also revealed that Days 2 through Retention of trace conditioning were all significantly less than Day 1.
2.3.5 DG and CA1 responding to the shock-US

Similar to the single neuron responses to the tone-CS, the DG and CA1 neurons showed an inverse pattern of responding to the US across training. Part A of Figure 2-6 shows the mean change in DG single neuron activity during the period 5-10 seconds after the delivery of the shock-US. Similar to the pattern of DG activity to the tone-CS, single neurons recorded during trace conditioning showed a greater increase in activity compared with pseudoconditioning on days 2 and 3 of acquisition. A repeated measures ANOVA showed a significant Training X Day X Block interaction, $F(4,702) = 2.48; p = 0.043$. Follow-up tests revealed a significant difference between trace and pseudoconditioning on Blocks 2 and 3 of Days 2 and 3. This second 5-sec period was analyzed in Figure 2-6A because DG responses to the shock-US were more sustained in the trace situation compared with the pseudoconditioning situation. This sustained response to the US during trace conditioning can be seen in the average histograms in Figure 2-3. The DG single neuron activity during the first 5-second period after the US is not shown; however, the first 5-second period showed a pattern of activity that was similar to the pattern in Figure 2-6A. Although the difference between the training conditions was slightly smaller for the first 5-second period after the US compared with the second, an analysis of this first 5-second period showed a Training X Block interaction, $F(2,776) = 5.26; p = 0.0054$. Follow ups showed a significant Training difference on Block 3 of Days 2 and 3.
Figure 2-6: Inverse DG and CA1 single neuron response patterns to the shock-US.

A. Mean change in DG single neuron activity from baseline in the 2nd 5-second period following the shock-US offset. B. Mean change in CA1 single neuron activity in the 1st 5-second period following US offset. Each point represents the mean standardized change in activity to the US in 2-trial blocks on each day of acquisition (Days 1-3). Error bars = S.E.M. Arrowhead on the ordinate axis shows the level of no change in activity.

The mean change in CA1 single neuron activity during the first 5-second period after the US is shown in Figure 2-6B. Analysis of these data showed no significant effects of Training. Analysis of the second 5-second period after the US also revealed no significant effects of Training (data not shown). Nevertheless, the pattern of decreasing CA1 responding to the US during trace conditioning was opposite to the increasing pattern of responding to the US for DG neurons in Figure 2-6A. This inverse DG and CA1 relationship was similar to the inverse pattern of responding to the CS shown in Figure 2-5.

Digital video was collected for each rat on each trial. Rats showed virtually no movement during the majority of trials, most likely due to the 4-day acclimation to the restrainer and recording apparatus. Three rats, however, showed some movement during several of the initial trials of the initial days of pseudoconditioning. All artifacts from
these movements were removed from the data. There were no noticeable movement responses to the CS. As expected, each rat showed a postural flinch response to the shock-US. The duration of this flinch response was approximately a second. Although all single neuron data were verified to be free of shock artifact and movement artifact due to the flinch response, it is possible that some of the single neuron responses to the US may have been related to the rats’ motoric flinch response, possibly through modulation from other motor brain areas.

2.3.6 DG and CA1 responding during the trace interval

Although DG and CA1 single neurons showed learning-related patterns of responding to the CS and US across training, there was very little if any learning-related responding during the trace interval. Figure 2-7 shows the mean change in DG and CA1 single neuron firing during the 5-second tone-CS period and the first 5 seconds of the trace interval (i.e., the 5-second period immediately after the tone-CS). The left panels show the same data as depicted in Figure 2-5 averaged into each day of training. These panels show a large increase in DG activity on Day 2 and Day 3, and a consistent reduction in CA1 activity across all days of trace fear conditioning. In contrast, the right panels of this figure show that there was no learning-related increase or decrease in DG or CA1 activity during the first 5-second period of the trace interval on any day of training. This is also exhibited by statistical analyses which revealed significant Training X Day effects for the DG and CA1 data in the left panels, $F(3,520) = 5.82; p = 0.0006$
and $F(3,403) = 3.88; p = 0.0093$, respectively; but no significant effects at all for the data in the right panels. Moreover, the three subsequent 5-second periods during the trace interval, which are not shown here, also showed no learning-related increases or decreases in activity whatsoever. Analyses conducted on these three subsequent 5-second periods also showed no significant effects or trends toward significance. This absence of learning-related activity during the trace interval can also be seen in the average histograms in Figures 2-3 and 2-4.
Analyses of changes in activity during the trace interval in Figure 2-7 showed no DG or CA1 learning-related encoding during the trace interval. These analyses are based on a firing rate code and may not be sensitive to patterned coding occurring during the trace interval. A series of analyses were therefore undertaken to determine if non-rate patterns of DG or CA1 single neuron activity might be encoding learning-related information during the trace interval. These analyses examined inter-spike interval (ISI) relationships and included an examination of the ISI distributions and average ISI histograms (Compte et al., 2003). In addition, the coefficient of variation of the ISI series during the trace interval was calculated for each single neuron to determine if the trace conditioning situation exhibited learning-related changes in ISI variability in response to the tone-CS (Compte et al., 2003). The entropy of the ISI distribution, which is a measure of the randomness of the ISI sequence, was also examined during the trace interval (Rogers, Runyan, Vaidyanathan, & Schwaber, 2001; Szucs, Pinto, Rabinovich, Abarbanel, & Selverston, 2003). This measure was used to determine if the sequence of ISIs in the trace condition became more patterned following the tone-CS compared with the pseudoconditioning situation. In sum, no ISI pattern or sequence analysis revealed significant effects for DG or CA1 activity during the trace interval. Autocorrelograms were also applied to the DG and CA1 single neuron activity during the various portions of the trial to determine if there was a learning-related presence of theta-patterned activity. The number of trials with theta-patterned activity was relatively low in CA1 (i.e., 13%) and DG (7.5%), and the presence of theta-patterned activity was not significantly different for trace and pseudoconditioning (p>0.05).
2.3.7 Inverse DG and CA1 relationship

Figures 2-5 through 2-7 suggest that DG and CA1 single neurons may encode trace conditioning information in an inverse manner. A series of Pearson correlation analyses were applied to these data in order to determine the relationship, if any, of DG and CA1 single neuron responding during trace fear conditioning.

A correlation analysis of the DG and CA1 single neuron responses to the tone-CS using the 2-trial block means in Figure 2-5 revealed a significant inverse correlation across the first 9 blocks (i.e., first 3 days of paired training), $r(7) = -0.709$, $p=0.023$. A similar level of significance was obtained by including the first CS-alone retention block, $r(8) = -0.614$, $p=0.046$; however, significance was eliminated if all CS-alone retention blocks were included in the analysis, $r(10) = -0.409$, $p=0.27$. A correlation analysis of the DG and CA1 single neuron responses to the shock-US using the 2-trial block means in Figure 2-6 did not reveal a significant correlation across the 9 blocks, $r(7) = -0.143$, $p=0.355$. Nevertheless, the correlation of DG and CA1 responding to the US was in the inverse direction, similar to the CS correlation measure.

The DG and CA1 mean neuronal responses in Figure 2-5 showed a significant inverse correlation suggesting that DG and CA1 showed an inverse overall pattern of DG and CA1 responding to the CS. A correlation analysis was also applied to the DG and CA1 single neuron responding on individual trials. This analysis used one mean response to the CS, US or trace interval per CA1 electrode and DG electrode within each animal on an individual trial. This analysis, and several other variations, did not reveal significant effects. Moreover, the largest correlations of DG and CA1 responses to the
tone-CS, trace interval, and US using this approach were all non-significant, \( r(51) = 0.155, p=0.21; r(49) = 0.109, p=0.21; \) and \( r(36) = 0.051, p=0.37, \) respectively. This suggests that there is an inverse complementary relationship between DG and CA1, not at the level of synaptic responding within a trial, but at the overall mean pattern of responding to the CS across blocks of trials. This also implies that there may be some variability in the DG and CA1 pattern of activity within a given trial, but DG and CA1 activity shows an inverse overall pattern of activity averaged across a 2-trial block. Analyses also correlated CS and US neuronal responses with changes in HR. These analyses used either maximum absolute HR responses as shown in Figure 2-2 or maximum bradycardia following the CS or US. These correlations of HR and DG or CA1 activity revealed no significant effects or trends toward significance. The largest correlations of HR with DG and CA1 activity were, \( r(74) = 0.047, p=0.36 \) and \( r(52) = 0.051, p=0.37, \) respectively.

2.3.8 Inverse DG and CA1 activity patterns conserved in slower firing rate ranges

All previous analyses were conducted on DG single neurons with baseline firing rates below 5 Hz and CA1 single neurons with baseline firing rates below 8 Hz. These criteria were selected based on the distributions of DG and CA1 baseline firing rates, and on criteria used in previous studies (e.g., Fox & Ranck, 1975; D. Nitz & McNaughton, 2004; D. A. Nitz & McNaughton, 1999). An additional set of follow-up analyses were conducted using the single neurons with much lower baseline firing rates. This was done to provide additional evidence that the patterns of DG and CA1 activity in the previous
figures were due to principal cells and not due to a handful of slow-firing interneurons. Thus, the same standard score analyses were repeated for single neurons with firing rates well below the principal cell firing rate. These analyses were conducted using only the DG cells firing in baseline below 2 Hz and CA1 cells firing in baseline below 4 Hz. This subset of units consists of 81% and 93% of all DG and CA1 units included in the previous figures and analyses. Parts A and B of Figure 2-8 show the mean change in standardized activity (z-scores) during the tone-CS for these low baseline firing DG and CA1 single neurons. Panel C shows the mean change in standardized activity during the shock-US for the low baseline firing DG single neurons. These low firing DG single neurons exhibited an increase in activity to the CS and US on days 2 and 3 of acquisition, a pattern of activity that was identical to Figures 2-5 and 2-6. A repeated measures ANOVA conducted on the DG response to the CS showed a significant Training X Day interaction, $F(3,375) = 3.0226; p = 0.030$. A repeated measures ANOVA conducted on the DG response to the US also showed a significant Training X Day interaction, $F(2,261) = 3.2838; p = 0.0390$. Follow-up tests revealed a significant difference between the Training conditions on Day 2 for both the CS and US activity. The CA1 single neurons with low baseline firing rates showed a decrease in activity across days, similar to the CA1 units shown in Figure 2-5. A repeated measures ANOVA conducted on this CA1 response to the CS showed a significant Training X Day interaction, $F(3,366) = 2.7059; p = 0.04519$. Follow-up tests revealed a significant difference between the Training conditions on the retention day. These data show that the learning-related inverse patterns of activity seen in DG and CA1 single neurons are due primarily, if not entirely, to the firing of principal cells.
Mean z-scores were calculated for DG and CA1 single neurons with very low baseline firing rates (i.e., below 2 Hz for DG and below 4 Hz for CA1). A. Mean change in single neuron activity from baseline during the 5-second tone-CS for low-firing DG single neurons. B. Mean change in single neuron activity during the tone-CS for low-firing CA1 single neurons. C. Mean change in single neuron activity during the 2nd 5-second period following the shock-US for low-firing DG single neurons. These are the same analyses shown in Figures 2-5 and 2-6 using only the low firing rate neurons. Each point represents the mean standardized change in activity during the 5-second response period in a 2-trial block on each day of acquisition (Days 1-3) and on CS-alone retention trials (Ret, panels A and B). Error bars = S.E.M. Arrowhead on the ordinate axis shows the level of no change in activity.
2.3.9 Robust vs. non-robust DG single neuron responses

The previous analyses in this chapter show that single neurons recorded from DG show significant increases in activity to both the CS and US during trace fear conditioning. A follow-up analysis was conducted to determine if the increases in DG responding were due to non-robust sparse increases in activity per trial (e.g., 0-2.5 mean spike increase in a 5-sec window), or if the increases in DG responding were due to robust increases in firing per trial (e.g., >2.5 mean spike increase in a 5-sec window). This level of less than 2.5 mean spike increase/trial was used in order to capture those neurons that might be producing a sparse discharge of only 1 or 2 spikes on a given trial. Such neurons may be silent cells that are active only in response to the CS or US (e.g., Fox & Ranck, 1975). The percentage of DG units firing an average of less than 2.5 spikes (non-robust) and an average of 2.5 spikes or greater (i.e., robust) above baseline per trial was calculated during the tone-CS or during the period following the shock-US on the last day of trace and pseudoconditioning (i.e., Day 3). Table 2-1 shows the percentage of DG single neurons recorded on Day 3 that exhibited significant z-scores (i.e., $z \geq 1.96$, $p<0.05$) and non-significant z-scores in each response category. This table shows that there was a fairly low percentage of neurons showing significant responding to the CS and US in the non-robust category. In fact, there was a greater percentage of responses to the CS during trace conditioning that were non-robust and non-significant (30.8%) compared with the responses that were non-robust and significant (12.3%). Moreover, all of the neurons firing robust responses to the CS and US contributed significant responses to the overall means (i.e., 0% robust non-significant). The table
also shows that the significant differences between trace and pseudoconditioning were due primarily to single neurons firing significant (p<0.05) and robust responses to the CS and US. Figure 2-9A shows four histograms of exemplar DG single neurons firing robust (i.e., 2.5 or more mean spike increase per trial) and non-robust responses (i.e., less than 2.5 mean spike increase per trial) to the CS and US.

### Table 2-1: DG single neurons showing firing increases to the CS and US on Day 3.

<table>
<thead>
<tr>
<th></th>
<th>Significant responses</th>
<th>Non-significant responses</th>
<th>Total responses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0-2.5 spikes</td>
<td>&gt; 2.5 spikes</td>
<td>0-2.5 spikes</td>
</tr>
<tr>
<td><strong>CS responses</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trace</td>
<td>12.3%</td>
<td>21.5%</td>
<td>30.8%</td>
</tr>
<tr>
<td>Pseudo</td>
<td>9.7%</td>
<td>11.3%</td>
<td>12.9%</td>
</tr>
<tr>
<td><strong>US responses</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trace</td>
<td>16.9%</td>
<td>24.6%</td>
<td>20.0%</td>
</tr>
<tr>
<td>Pseudo</td>
<td>16.1%</td>
<td>6.5%</td>
<td>19.4%</td>
</tr>
</tbody>
</table>

Percentages based on total number of DG single neurons recorded on Day 3: Trace n = 65; Pseudo n = 62. Significant responses based on mean z-scores for each unit (4-trial block; p < 0.05). a: Trace significantly different than Pseudo, p < 0.05; b: p < 0.01
Figure 2-9: Example single neuron responses to the CS and US.

Each histogram shows the number of spikes/500-ms bin, summed across trials 1-6 for an example single neuron recorded during trace fear conditioning. **A.** Four example DG single neurons that showed an increase in responding to the CS or US during acquisition. Both robust responses (≥2.5 mean spikes increase/trial) and less robust (< 2.5 mean spike increase/trial) responses were found in DG single neurons. All of these example single neurons exhibited a baseline firing rate less than 1.0 Hz. **B.** An example CA1 single neuron that showed an excitatory response to the CS early in acquisition and another example CA1 single neuron that showed a decrease in activity to the CS on the retention day. These CA1 neurons exhibited a baseline firing rate less than 2.0 Hz. Solid arrowheads mark the latency of US delivery on acquisition trials. A 20-second trace interval separates the CS and US on paired trials. No USs were delivered on CS-alone retention trials following trace fear conditioning, but the open arrowhead marks the latency of US delivery on previous training trials.

It is also highly unlikely that the increases in DG single neuron activity reported in the previous analyses in this study were due to very low firing rate neurons that showed only one or two spikes following the CS or US. First, there was a relatively
small number of single neurons with very low spontaneous firing rates encountered in DG and CA1. Only 13.0% of the DG neurons and 7.9% of the CA1 neurons had background firing rates below 0.1875 Hz. This firing rate represents approximately 3 spikes during the 16 second baseline period. Typical z-score calculations with background firing rates below 0.1875 Hz begin to inflate the z-score calculation. The calculations used in our z-score analyses, however, used a correction factor that prevented cells with very low baseline variability to overly influence the mean z-scores. Thus, the modest number of very low firing rate neurons recorded and the z-score correction makes it highly unlikely that low firing rate neurons provided an excessive contribution to the group means of increased activity. Furthermore, a correlation analysis shows there was no relationship between background firing rate and z-score response magnitude for DG (r=0.007), nor for CA1 (r=0.124) single neurons. Both correlations were not significant at the p<0.05 level, providing additional evidence that low firing rate neurons did not provide a biased contribution to the group means of single neuron responding.
Figure 2-10: Example single neuron responses to the CS and US.

Each histogram shows the number of spikes/500-ms bin, summed across trials 1-6 for an example single neuron recorded during trace fear conditioning. A. Four example DG single neurons that showed an increase in responding to the CS or US during acquisition. Both robust responses ($\geq 2.5$ mean spikes increase/trial) and less robust ($< 2.5$ mean spike increase/trial) responses were found in DG single neurons. All of these example single neurons exhibited a baseline firing rate less than 1.0 Hz. B. An example CA1 single neuron that showed an excitatory response to the CS early in acquisition and another example CA1 single neuron that showed a decrease in activity to the CS on the retention day. These CA1 neurons exhibited a baseline firing rate less than 2.0 Hz. Solid arrowheads mark the latency of US delivery on acquisition trials. A 20-second trace interval separates the CS and US on paired trials. No USs were delivered on CS-alone retention trials following trace fear conditioning, but the open arrowhead marks the latency of US delivery on previous training trials.
2.3.10 *Inverse DG-CA1 activity patterns are exhibited in rats that did not receive pseudoconditioning*

The previous analyses of learning-related single neuron activity in this study were obtained using within-subjects group comparisons. Each rat first received 4 days of acclimation to the conditioning chamber and restraining apparatus, 4 days of pseudoconditioning, a day of rest, then 4 days of trace fear conditioning. This raises the possibility that the differences between trace and pseudoconditioning obtained in the previous analyses were the result of habituation or a change in the novelty of the stimuli across the 13 days of training. Moreover, it is possible that DG and CA1 single neuron changes in activity during trace fear conditioning were not learning-related responses to the CS and US, but were due to changes in the novelty of the stimuli used in unpaired pseudoconditioning versus paired trace conditioning. This issue was addressed by adding a group of rats to this study that did not receive unpaired pseudoconditioning. This extra group of rats provided additional evidence that the changes in DG and CA1 activity in the previous analyses were learning-related and not due to a change in the novelty of the stimuli across the 13 days of training.

Similar to the other rats in this study a group of eight rats were implanted with the DG-CA1 recording array. Following recovery these rats received no acclimation to the restraining and recording chamber, but instead received two days of CS-alone habituation/acclimation trials (6-10 trials/day) followed by two days of 20-second trace fear conditioning (6-10 trials/day). Moreover, the first time that these rats received the CS and US in the same session was in the trace fear conditioning session. The location of the DG and CA1 electrodes was similar to those shown in Figure 2-1. An average of 17
DG and 41 CA1 single neurons were recorded on each day of trace fear conditioning. Figure 2-10 shows the mean change in DG and CA1 single neuron activity averaged across 6 trace fear conditioning trials. The DG single neurons showed an increase in activity to the tone-CS and shock-US on Day 2 compared with Day 1 and CA1 single neurons showed a decrease in activity to the tone-CS on Day 2 compared with Day 1. This inverse pattern of activity is similar to the pattern observed in the 6 within-subjects rats shown in the previous analyses. A two-way ANOVA with factors Region (DG and CA1) and Day (Days 1 and 2) on the CS response showed a significant Region X Day interaction F(1,112) = 15.52; p = 0.000142. Follow-up analyses revealed a significant difference between days for both Regions. There was also a significant difference between Regions on Day 2. A similar two-way ANOVA on the US response showed a significant Region X Day interaction F(1,112) = 8.05; p = 0.0054. Follow-up analyses revealed a significant difference between days for DG and a significant difference between Regions on Day 2. These results provide additional evidence that the inverse pattern of activity between DG and CA1 single neurons is learning-related and not due to habituation or changes in novelty across repeated days of training.
Single neurons were recorded in the DG and CA1 of 8 separate rats that did not receive pseudoconditioning training prior to trace fear conditioning. These rats received two days of habituation/acclimation trials prior to receiving trace fear conditioning. Graphs depict the mean change in single neuron activity to the tone-CS and shock-US for DG (left panels) and CA1 (right panels) single neurons. Each bar represents the mean standardized change in activity averaged across the first 6 trials on Days 1 and 2 of 20-second trace fear conditioning. The DG and CA1 single neurons recorded from these 8 rats exhibited inverse response patterns of activity. This pattern of activity is consistent with the 6 rats that received pseudoconditioning training prior to trace fear conditioning. Error bars = S.E.M.

2.3.11 No time-locked single neuron firing

Trace fear conditioning in rabbits has been shown to produce phasic increases in CA1 single neuron activity on CS-alone retention trials that are time-locked to the latency when the shock-US had been delivered on previous training trials (McEchron, Tseng, & Disterhoft, 2003). A similar set of timing analyses was undertaken in this study to determine if DG or CA1 single neurons in rats also show timed encoding of the trace interval on CS-alone retention trials. For each single neuron in the present study, the activity following the tone-CS in one-second bins was used to determine the latency at
which each neuron exhibited maximal firing. On CS-alone trials following trace fear conditioning, there was no significant difference in the percentage of single neurons firing maximally at any given latency compared with pseudoconditioning. Similar results were obtained using a non-binning Gaussian kernel density analysis to find the latency of maximal firing. These results suggest that DG and CA1 single neurons in the rat hippocampus do not consistently exhibit the timed neuronal responses that were observed in the rabbit CA1 single neurons (McEchron, Tseng, & Disterhoft, 2003).

2.4 Discussion

Single neuron activity was recorded simultaneously in the DG and CA1 areas of the rat hippocampus during unpaired pseudoconditioning and subsequent trace fear conditioning. This study showed that hippocampal single neurons encode information about the CS and US, but not about the trace interval. This result was surprising because lesion studies showed that the hippocampus is necessary for fear and eyeblink conditioning tasks only when the CS and US are separated by the trace interval (Chowdhury, Quinn, & Fanselow, 2005; McEchron, Bouwmeester, Tseng, Weiss, & Disterhoft, 1998; McEchron, Tseng, & Disterhoft, 2000; Moyer, Deyo, & Disterhoft, 1990; Phillips & LeDoux, 1992; Quinn, Oommen, Morrison, & Fanselow, 2002; Solomon, Vander Schaaf, Thompson, & Weisz, 1986). This critical role of the hippocampus in trace conditioning paradigms has led some computational models to suggest that hippocampal neurons may provide some form of sustained activity that can bridge across the trace interval. The bridging activity would then provide a Hebbian-like
overlap in CS and US neuronal activity that would allow associative changes in plasticity to occur (e.g., Rodriguez & Levy, 2001; Wallenstein, Eichenbaum, & Hasselmo, 1998). The present study used rate and pattern analyses, and showed that overall there was no learning-related DG or CA1 activity that bridged the trace interval. We suggest that other areas must be providing bridging activity during the trace interval. One area that is known to provide sustained tonic levels of activation during temporal intervals is the medial prefrontal cortex (e.g., J. Y. Chang, Chen, Luo, Shi, & Woodward, 2002; Fuster, 1973, 1990). This area receives dense projections from both the hippocampus and the amygdala (Chiba, 2000; Jay & Witter, 1991). A recent study by Helmstetter’s group used functional magnetic resonance imaging in humans to examine brain patterns of activity during trace fear conditioning (Knight, Cheng, Smith, Stein, & Helmstetter, 2004). They showed that learning-related activation of the hippocampus occurs primarily in response to the CS and US but does not occur during the trace interval. In contrast, frontal cortex regions in their study exhibited learning-related activation during the trace interval. Baeg et al. (2001) have described medial prefrontal cells in the rat that increase and tonically maintain their activity across a 2-second trace interval during trace fear conditioning. Other work has shown that the prefrontal cortex is necessary for trace eyeblink conditioning (Weible, McEchron, & Disterhoft, 2000), and that extracellular signal-regulated kinase phosphorylation in the prefrontal area may be necessary for the retention of trace fear conditioned memories (Runyan & Dash, 2004; Runyan, Moore, & Dash, 2004). Chapter 3 will examine single neuron activity in the medial prefrontal cortex during trace fear conditioning to determine whether neurons in this structure exhibit increases in activity that could bridge the trace interval.
Chapter 2 showed that single neurons recorded from the DG and CA1 regions of the hippocampus encoded information about the CS and US. Single neurons recorded from the DG exhibited learning-related increases in activity to the CS and US early in trace conditioning, which continued throughout training. The CA1 neurons recorded from the same animals showed an opposite pattern of responding to the CS and US during trace fear conditioning. Correlation analyses using overall averages showed that there was an inverse relationship between DG and CA1 single neuron responding to the CS across trials; however, other correlation analyses within individual trials did not show any other relationship between DG and CA1 responses. A separate group of animals that received no early pseudoconditioning showed the same inverse pattern of DG and CA1 single neuron responding to the CS and US during trace fear conditioning. This suggests that neuronal responses in DG have an inverse relationship with the responses in CA1 as an overall mean pattern across trace fear conditioning, but not at discrete time points during an individual trial.

The inverse relationship of DG and CA1 encoding of learning-related information may be indirectly supported by a number of computational models of hippocampal function. Several models suggest that the function of DG neurons may be to encode patterns of multiple sensory inputs during a learning episode and transmit this information to downstream areas of the hippocampus, such as CA3 and CA1 (McClelland, McNaughton, & O'Reilly, 1995; N. McNaughton & Morris, 1987; Rolls, 1996). Other models suggest that CA1 neurons may serve as a comparator of inputs from the entorhinal cortex and DG and CA3 areas, and this comparison may serve to distinguish familiar and novel patterns (Hasselmo, Fransen, Dickson, & Alonso, 2000).
Together these models may provide a meaningful interpretation of the results of the present study. Specifically, the single neurons recorded in DG in this study showed large learning-related increases in activity to the CS and US early on Day 2 of paired trace fear conditioning. These increases in activity to the CS and US continued throughout paired training, and may have served to establish or maintain the associative strength of the individual learning-related stimuli (i.e., a qualitative “pattern” of the CS and US). The DG responding to the CS diminished back to a non-associative level after several non-reinforced CS-alone retention trials. These diminished responses may reflect an encoded decrease in the associative strength of the CS. The CA1 single neurons in the same rats showed an inverse pattern of activity compared with the DG neurons. The CA1 neurons showed a very early increase in responding to the CS on Day 1 when the unfamiliar CS-trace-US pattern of stimuli was first presented. This CA1 neuronal response to the CS, and somewhat to the US, then diminished and became a decrease in activity on subsequent days of training as the CS-trace-US pattern of trace fear stimuli decreased in novelty. Thus, the CA1 neurons may have served to distinguish familiar and novel patterns of fear-related stimuli (i.e., the CS and US).

A number of electrophysiological studies also support the notion that DG and CA1 have distinct or inverse roles in learning. A study by Wiebe and Staubli (1999) recorded single neuron activity from DG and CA1 during an odor-guided delayed nonmatch-to-sample task with an initial sample and subsequent test phase. This study showed that cells in CA1 rather than DG showed much more encoding of odor identity during the early sample odor phase, while the DG more so than CA1 showed odor-selective activity during the subsequent test phase. These results are analogous to the
model where CA1 assesses the novelty and familiarity of the training context, while the DG neurons assess a pattern of sensory inputs. McNaughton’s group has also performed single neuron recordings simultaneously from the DG and CA1 of rats during exploration. One of their studies performed manipulations in the novelty of exploratory environments and showed that CA1 principal cells exhibited an increase in firing in the novel environments compared with the familiar ones (D. Nitz & McNaughton, 2004). The DG principal cells, on the other hand, did not show this pattern, but did show a small trend toward increased firing in familiar environments. Again, this is consistent with the idea that DG neurons may be encoding stimulus patterns, while CA1 neurons distinguish between familiar and novel stimuli.

Other studies support the notion that DG and CA1 have distinct roles in learning. A study by Kesner’s group has shown that lesions placed in the rat DG impair a delayed-match-to-sample for spatial location task, but lesions placed in CA1 had no effect on this task (Gilbert, Kesner, & Lee, 2001). This same study also showed that rats with CA1 lesions were impaired in a spatial temporal order version of the eight-arm radial maze, but DG lesions did not impair this spatial temporal order task. A study by Weitemier and Ryabinin (2004) used the inducible transcription factor Zif268 to show that the DG rather than CA1 and CA3 was the primary area of the hippocampus that was activated in a trace fear conditioning recall procedure, but this pattern of activation was not seen in a non-hippocampus-dependent delay fear conditioning recall procedure.

Recently, McEchron et al. (2003) recorded single neuron activity from the CA1 area of the rabbit hippocampus during trace fear conditioning with a 10- or 20-second trace interval (McEchron, Tseng, & Disterhoft, 2003). Rabbits in this recent study
received a single day of training followed by a second day of CS-alone trials. There are some differences between the results of this recent rabbit study and the results obtained in the present within-subjects rat study. The rabbit study showed no consistent learning related changes in CA1 activity during the CS, trace, or US during paired trials or retention trials. The present study, however, showed a small increase in CA1 responding to the CS on day 1. More importantly, the CA1 response to the CS developed into a significant learning-related decrease in activity across the subsequent 3 days of training. It is likely that this decrease in CA1 responding could not be detected in the previous rabbit study because the subjects received only a single day of training that did not allow enough daily training sessions for the development of the response decrease. The McEchron et al. (2003) rabbit study also showed that there were learning-related phasic increases in activity on CS-alone trials timed to the duration of the trace interval. The results from the present study showed that there was no significant difference between the trace and pseudoconditioning situations in the number of DG or CA1 neurons that fire maximally at any given latency. There are several possible explanations for the absence of significant timed trace interval encoding in the present rat hippocampal recording study. This study used a within-subjects pseudoconditioning procedure prior to paired trace fear conditioning, and rats received fewer daily training trials (6-10 trials in the present study compared with 35 in the rabbit study). It is possible that these design factors did not allow the development of a hippocampal code of trace interval duration. It is also possible that rabbits have a greater capacity to attend to and encode the temporal pattern of the trace fear conditioning trial compared with rats. This may be especially
true for the semi-restrained preparation used in the present study which can produce more stress for rats compared with rabbits.

The present study shows that trace fear conditioning information is encoded by DG and CA1 neurons in an inverse manner. It is probable that other trace conditioning paradigms, like trace eyeblink conditioning, will show a similar inverse pattern of DG and CA1 activity during training. To our knowledge, DG single neuron activity has not been examined extensively in other trace conditioning paradigms. Single neuron responses in CA1 during trace eyeblink conditioning have shown a pattern of activity similar to those in this study: increases to the CS and US early in training followed by decreases to these stimuli during later phases of training when conditioning became asymptotic (McEchron & Disterhoft, 1997). On the other hand, it is possible that trace fear and trace eyeblink conditioning may place different neuronal processing demands on the hippocampus. Moreover, it is possible that the results of the present study are specific to non-escapable trace fear conditioning rather than escapable trace eyeblink conditioning. There are some reports that dorsal hippocampal lesions may produce only moderate impairments in trace eyeblink conditioning. A study by Port and colleagues showed that hippocampal lesions produce different impairments in the timing of trace conditioned eyeblink responses for more aversive shock-USs compared with airpuff-USs (Port, Romano, Steinmetz, Mikhail, & Patterson, 1986; but see Takehara, Kawahara, Takatsuki, & Kirino, 2002; and Tseng, Guan, Disterhoft, & Weiss, 2004). There may also be different hippocampal demands for these trace conditioning paradigms because of the duration of the trace intervals used in the two paradigms. The trace interval in trace
fear conditioning can be 10 to 30 times longer than the trace interval in trace eyeblink conditioning.

The behavioral data in the present study suggests that learning-related HR fear responses emerge on the first day of trace fear conditioning. The single neuron activity in CA1 shows an increasing trend on this first day of trace fear conditioning, but the significant increases in activity in DG do not occur until the second day of trace fear conditioning. This may suggest that the initial association of the CS and US is related more to the neural processing in CA1 rather than in DG. This may also indicate that there are several neural stages of trace fear conditioning. For example, the initial low-level association of the CS and US may occur on day 1, while a more complex assessment of the CS, US, and their relationship may occur on day 2. Thus, the increases in activity to the CS and US recorded in DG may be related to a learning-related event which follows the initial CS-US association. If this is true the HR conditioned response may not be sensitive enough to distinguish these different phases of learning, as the trace conditioned HR responses in this study showed a fairly uniform increase across each day of trace fear conditioning. It is also possible that the initial neural events which precede the emergence of the trace fear conditioned response do not occur in DG or CA1. For example, these precursor neural events may occur in CA3 as proposed in Rodriguez and Levy (2001).

A number of studies have used the trace fear conditioning paradigm to examine the role of the hippocampus in learning and memory. Tonegawa and colleagues showed that NMDA receptors in the CA1 area of the hippocampus are necessary for learning trace fear conditioning (Huerta, Sun, Wilson, & Tonegawa, 2000). Another study
demonstrated that inhibiting neurogenesis specifically impairs trace fear conditioning in the rat while leaving other hippocampus-dependent tasks relatively intact (Shors, Townsend, Zhao, Kozorovitskiy, & Gould, 2002). Several recent studies in rats and mice have shown that trace but not delay fear conditioning is impaired by aging (Blank, Nijholt, Kye, Radulovic, & Spiess, 2003; McEchron, Cheng, & Gilmartin, 2004; Moyer & Brown, 2006). A recent study from our laboratory showed that nutritional iron deficiency during early development permanently impairs trace fear conditioning in rats, an effect that may be mediated by reduced hippocampal transmission (McEchron, Cheng, Liu, Connor, & Gilmartin, 2005; McEchron & Paronish, 2005). The trace fear conditioning paradigm has also been used in humans to examine the pattern of hippocampal activation compared with other brain areas like the amygdala (e.g., Buchel, Dolan, Armony, & Friston, 1999; Knight, Cheng, Smith, Stein, & Helmstetter, 2004).

This chapter provides important information about sub-region-specific hippocampal processing of information during trace fear conditioning. The results summarized in this chapter showed that DG and CA1 neurons encode primarily learning-related information about the CS and US, and that DG and CA1 neurons encode learning-related information using inverse patterns of activity across the course of learning. The results also showed that single neurons in the DG and CA1 hippocampus do not exhibit activity patterns that bridge the trace interval. Chapter 3 will test the hypothesis that single neurons in the medial prefrontal cortex, an important part of the trace fear network, encode the trace interval in trace fear conditioning.
Chapter 3

Single Neurons in the Medial Prefrontal Cortex of the Rat Exhibit Tonic and Phasic Coding during Trace Fear Conditioning

3.1 Introduction

Chapter 2 showed that single neurons in two sub-regions of the hippocampus exhibit specific patterns of learning-related activity to the CS and US during trace fear conditioning; however, these areas of the hippocampus showed very little if any changes in activity during the 20-sec trace interval (Gilmartin & McEchron, 2005a). Computational models suggest that some brain area(s) must provide sustained bridging activity during the trace interval to allow a Hebbian-like overlap of CS and US information (Levy, Sanyal, Rodriguez, Sullivan, & Wu, 2005; Rodriguez & Levy, 2001; Wallenstein, Eichenbaum, & Hasselmo, 1998). Thus, the results reviewed in Chapter 2 suggest that parts of the trace fear network outside of the hippocampus must be providing important bridging information during the trace interval. The present study sought to determine if another structure in the trace fear network, the medial prefrontal cortex (mPFC), provides a bridging signal during trace fear conditioning. This study was published in 2005 (Gilmartin & McEchron, 2005b).

The mPFC receives projections from both the hippocampus and the amygdala (Chiba, 2000; Jay & Witter, 1991) and several studies suggest that it is necessary for both trace fear and trace eyeblink conditioning (McLaughlin, Skaggs, Churchwell, & Powell,
2002; Runyan & Dash, 2004; Runyan, Moore, & Dash, 2004; Weible, McEchron, & Disterhoft, 2000). Although no studies have examined the activity of mPFC neurons during 20-s trace fear conditioning, several neurophysiology studies suggest that this structure may play a role in sustaining activity during the trace interval that separates the CS and US. For example, delayed matching-to-sample studies have shown that single neurons in the mPFC exhibit tonic firing responses during empty intervals separating stimuli and behavioral responses (J. Y. Chang, Chen, Luo, Shi, & Woodward, 2002; Fuster, 1973, 1990). Another study used the trace fear conditioning paradigm with a short 2-s trace interval and showed that mPFC neurons can show some level of sustained firing during the trace interval (Baeg et al., 2001). A recent study by Helmstetter and colleagues used functional magnetic resonance imaging during human trace fear conditioning and showed that frontal cortical regions were activated during the trace interval separating the CS and US (Knight, Cheng, Smith, Stein, & Helmstetter, 2004). Their study also showed that the hippocampus was activated during the CS and US but not during the trace interval, similar to the single neuron recording study in Chapter 2 (Gilmartin & McEchron, 2005a).

The present study examined single neuron activity in the prelimbic and infralimbic regions of the rat medial prefrontal cortex during trace fear conditioning with a 20-s trace interval. This allowed us to determine if single neurons in the mPFC: 1) encode learning-related information during trace fear conditioning; and 2) exhibit sustained bridging responses during the trace interval that separates the CS and US.
3.2 Materials and Methods

3.2.1 Subjects and surgery

Surgery was performed on a total of 21 adult female BN/F344 (n = 13) and Sprague-Dawley (n = 8) rats in this study (200-400 g; Harlan, IN). All rats were housed individually and received food and water *ad libitum*. All surgical procedures were in accordance with the National Institutes of Health and the Pennsylvania State University Institutional Animal Care and Use Committee. Rats were anesthetized with 1-2% halothane in 100% O₂ during surgery. Eyes were kept moist with antibacterial ophthalmic ointment. Each rat was positioned in a stereotaxic frame and the skull was exposed. Five self-tapping screws (#0-4.75mm, Small Parts, Inc., FL) were inserted ~1 mm into the skull to anchor the final dental cement head assembly. A 3-mm diameter hole was drilled in the skull above the left medial prefrontal cortex, and an 8-electrode array was stereotaxically lowered into the mPFC. The array consisted of eight Teflon-coated stainless steel electrodes arranged in two rows of 4 electrodes (50 µm diameter each; tip separation: 200-400 µm; NB Labs, Dennison, TX). One row was cut 0.75 mm longer than the other. This array configuration resulted in the short row of electrodes being positioned in the prelimbic area and the longer row in the infralimbic area of mPFC. Single neuron activity was monitored from two prelimbic and two infralimbic channels while the electrodes were being lowered in order to achieve optimal placement. Dental cement was used to secure the electrodes to the skull and close the remaining wound area. Skull screws were used as the ground and reference electrode.
3.2.2 Heart rate fear conditioning

Prior to training, the rats received four consecutive days of acclimation to the restrainer and conditioning chamber (20 minutes/day) in order to reduce arousal and struggling during restraint and testing. No stimuli were presented during the acclimation sessions. During acclimation, training, and testing sessions, rats were restrained as described in Chapter 2. Electrocardiographic (EKG) activity was recorded across two leads placed on the chest or across leads placed at the neck and lower back. The EKG leads were either stainless steel wound clips or mesh wires pressed to the skin with conductive gel. The EKG activity was amplified 10,000X, filtered between 10 and 1000 Hz, and sampled at 25 kHz. The ends of copper tubes (5 mm diameter) were placed 1 cm from the opening of each ear and served to deliver the auditory tone-CS (5 s; 6000 Hz; 80 dB). Two flexible wire mesh leads were secured to the tail of the rat, which served to deliver the shock-US (0.8 s; 0.75-mA alternating current).

One day after the fourth acclimation session, each rat received either three consecutive days of trace fear conditioning (Trace group, n = 11) or three consecutive days of unpaired training (Unpaired group, n = 10). All rats then received one session of CS-alone retention trials 24 hours later. All but two rats also received an additional session of CS-alone retention trials 24 hours after the first CS-alone session.

A total of 11 rats received three consecutive days of 20-s trace fear conditioning. Each 20-s trace fear conditioning session consisted of 6-8 paired CS-US trials using an intertrial interval (ITI) of 200 ± 20 s, in which the offset of the tone-CS was separated from the onset of the shock-US by an empty 20-s trace interval on each trial. For several
animals restraint stress caused struggling after 6 or 7 CS-trace-US trials on several of the training days. For these cases training was halted on this day, and additional trials were delivered on the following day of training. Twenty-four hours after the third trace fear conditioning session, rats received 10 CS-alone retention trials. Each rat received 1 paired CS-trace-US reminder trial before the CS-alone retention trials. Twenty-four hours after the CS-alone retention session, rats received an additional 10 CS-alone retention trials. No paired trial was delivered on the second retention day.

A total of 10 rats received three consecutive days of unpaired pseudoconditioning trials. The unpaired sessions were used to determine the non-associative level of behavioral and neuronal responding. Each unpaired session consisted of 6-8 CS-alone trials and 6-8 US-alone trials (ITI = 100 ± 20 s). During each unpaired session, the same stimulus was never presented more than two consecutive times. One day after the last unpaired session, rats received one US-alone reminder trial followed by 10 CS-alone trials. A second session of 10 CS-alone trials was delivered 24 hours later. No US-trial was delivered on the second retention day.

3.2.3 Single neuron recording

Single neuron analog signals were amplified (10,000X), filtered (300 Hz to 5 kHz), and collected with a DT-3100 Data Translation board (Data Translation, Marlboro, MA) attached to an 1.5-GHz computer, which sampled each channel at 25 kHz. The same computer sampled EKG activity and controlled the delivery of all stimuli. Single
neuron and EKG data were collected from 16 s before CS onset to 16 s after US onset using software developed in our laboratory.

Individual single neurons on an electrode were isolated using template-matching algorithms as described in Chapter 2.2.3. It is important to note that the configuration of single neurons on an electrode changed from one day of recording to the next in almost all cases. For this reason, each day’s recorded single neurons were treated as a new set of neurons.

3.2.4 Analyses

All statistical analyses were performed with the aid of Microsoft (Redmond, WA) Visual Basic routines developed in our laboratory, Minitab Statistical Software version 10.0 (State College, PA), and Statistica software version 6.0 (Tulsa, OK). Single neuron background firing rates were determined by calculating the mean discharge rate of each neuron during the 16-s baseline period before CS-onset on each trial. Changes in single neuron firing to the CS, trace interval, and US were measured on each trial using standard z-test change scores. The z-scores were calculated by subtracting the average baseline activity from the activity in a 1- or 5-s window during the trial. This difference in activity was then divided by the baseline variability. For neurons with very low baseline firing rates (i.e., < 0.10 Hz), the z-score was equivalent only to the difference score, and not divided by the baseline variability. This reduced the possibility that very low firing neurons would contribute inflated z-score values (see Chapter 2.2.4; Gilmartin & McEchron, 2005a).
The $z$-scores were used to assess the magnitude of increase or decrease in firing during a specific time window during the trial. The $z$-scores could be averaged across trials or within a group of neurons. In most cases, these $z$-scores compared the activity in 5-s periods during the CS, trace interval, or post-US period with the mean activity during the 16-s baseline. Our previous work suggests that standard scores of a small subset of single neuron data can become inflated by bursting responses or fluctuations in background firing rate (e.g., Chapter 2.2.4; Gilmartin & McEchron, 2005a). In order to reduce the possibility that a small number of overly active neurons can skew the overall group average, single neuron $z$-scores that were greater than 3 standard deviations from the mean were assigned a $z$-score equal to 3 standard deviations. Less than 3% of the standard score data were limited or capped with this method. Group comparisons of standard scores were performed using factorial repeated-measures ANOVAs which included the following factors: a repeated measure of 2-trial blocks within each day; a between factor of 5 days of training (i.e., 3 acquisition + 2 retention days); and a between factor of training condition (trace vs. unpaired). Due to the possible changes in neuronal sampling from one day of recording to the next, the trial-blocks factor was the only repeated measure used in these analyses. Moreover, the number and configuration of single neurons recorded from each individual electrode showed slight variations from one day of recording to the next in each animal; however, within each day the same single neurons were tracked accurately across the recording session. These small daily variations in the neuronal configuration on an electrode in most cases prevented accurate tracking of the same single neuron across multiple recording sessions. As a result, all single neuron analyses were based on the assumption that the population of single
neurons for a given experimental group was relatively homogenous in each region (prelimbic or infralimbic) of the mPFC during a specific stage of learning (i.e., day of training). Using this approach a sufficient sample size of neurons from an individual neuroanatomical region should reveal a consistent pattern of changes in activity across days of training for a given experimental training condition. Similar methodological approaches have been used in other studies that record single neuron activity across multiple days of training (Frank, Stanley, & Brown, 2004; Gilmartin & McEchron, 2005a; McEchron & Disterhoft, 1997; McEchron, Weible, & Disterhoft, 2001).

In some instances, the number of neurons was greater in the unpaired group compared with the paired group. All ANOVA calculations were performed using Statistica v6.0 (Tulsa, OK) which utilized a Type III Sums of Squares, which corrects for unequal sample sizes (e.g., Milliken & Johnson, 1984; Searle, 1987; Shaw & Mitchell-Olds, 1993). A Duncan’s multiple-range post-hoc test was used to test the significance of mean differences. Proportions of neurons were compared between groups using a non-parametric z-ratio-two-sample test of independent proportions (described by Rosner, 1990). An $\alpha$ level of 0.05 was required for significance in all analyses.

Average histograms were constructed using normalized firing rates. For each single neuron, action potentials were summed across 6 trials into 1-s bins. Activity in each bin was then normalized to the mean and standard deviation of the 16-s basal firing rate. Average histograms were then constructed by averaging the normalized activity in each bin across groups of neurons.

Software routines developed in our laboratory measured HR by calculating the time between the R-peaks in the raw EKG waveform and calculating beats per min in
each second. Changes in HR were analyzed using repeated measures ANOVAs, with an
$\alpha$ level of 0.05 required for significance. All HR records containing arrhythmias, high
baseline variability, or excessive movement artifact were excluded from analyses. These
criteria resulted in less than 5% of the total trials being excluded from the final analyses.

3.2.5 Histology

Marking lesions were placed at the tips of all electrodes by passing direct current
(20 $\mu$A) for 15 s. Rats were deeply anesthetized with 5% halothane in 100% $O_2$,
sacrificed by decapitation, and the brains were placed in a 10% formalin solution (0.9%
saline). Brains were then frozen, sectioned coronally, mounted on glass slides, and
stained with neutral red. A light microscope was used to locate electrode tips.

3.3 Results

3.3.1 Electrode placement

Neuronal and behavioral analyses were conducted using 21 rats which exhibited
reliable single neuron recordings from the mPFC during trace or unpaired sessions.
Analyses of single neuron activity were conducted using neurons that were recorded from
electrodes placed in either the prelimbic or infralimbic areas of mPFC. Figure 3-1 shows
the location of the electrode tips placed correctly in the prelimbic and infralimbic areas.
An average of 61 prelimbic neurons (range: 51-75) and 37 infralimbic neurons (range:
25-46) were recorded on each training session during trace fear conditioning. An average of 92 prelimbic neurons (range: 87-97) and 39 infralimbic neurons (range: 34-47) were recorded on each training session during unpaired training.

Figure 3-1: Electrode placements in the medial prefrontal cortex.

Coronal diagrams show the locations of the electrode tips in the prelimbic (filled circles) and infralimbic (open circles) areas of the medial prefrontal cortex (mPFC). The infralimbic region is immediately ventral to the prelimbic region and it is not present in the rostral-most section. Reprinted from The Rat Brain in Stereotaxic Coordinates, 4th ed., G. Paxinos and C. Watson, pp. 291–294, Copyright 1998, with permission from Elsevier.

3.3.2 Heart rate fear conditioning

Decreases in HR (i.e., bradycardia) to the CS were used to indicate conditioned fear responses. Overall the trace fear conditioning group showed large bradycardiac conditioned fear responses to the auditory-CS while the unpaired group showed no
conditioned fear responses. Analyses calculated changes in HR from baseline in 1-s bins averaged across the first 6 CS-trace-US trials of each session of trace conditioning or the first 6 CS-alone trials of each session of unpaired training. Part A of Figure 3-2 shows that rats developed a large decrease in HR during the CS and trace interval on day 1 of trace fear conditioning. This bradycardiac response was stronger on days 2 and 3 of trace conditioning and was also present on the CS-alone retention sessions. In contrast, rats in the unpaired group showed an increase in HR during the CS and a return to baseline levels at CS-offset. Part B of Figure 3-2 shows the mean change in HR during the second half of the trace interval across days of training. Analyses of the HR changes in this figure revealed a significant Training Group X Day interaction, $F(4,52) = 3.60, p = 0.0115$. Follow-up analyses showed a significant difference between the training groups on days 2-3 of acquisition and on both CS-alone retention days. Although not shown here, analyses of the changes in HR during the CS showed a nearly identical group difference and function across days $F(4,52) = 6.27, p = 0.00034$. 
3.3.3 *mPFC average single neuron responses to CS and US*

Single neurons in the prelimbic and infralimbic regions of the mPFC showed inverse patterns of responding to the CS and US during trace fear conditioning. Figure 3-3 shows the average normalized peri-event time histograms for all prelimbic and infralimbic single neurons recorded on the last day of trace and unpaired conditioning (i.e., day 3). This day of training is depicted because it shows the largest changes in learning-related activity; however, the other days of training showed similar patterns of activity. The average prelimbic response showed a greater increase in activity during the CS and following the US in the trace conditioning group compared with the unpaired
control group. In contrast, the infralimbic neurons showed a decrease in activity during the CS and US in the trace group but not in the unpaired group. Prelimbic neurons also exhibited elevated firing during the trace interval or post-CS period. This elevated firing was seen in both the trace and unpaired groups, but was much stronger in the trace group. On average the infralimbic neurons showed little or no change in firing during the trace interval.
Figure 3-3: *Average peri-event time histograms for medial prefrontal single neurons.*

Histograms show the mean normalized firing rate for all prelimbic and infralimbic single neurons recorded on Day 3 of trace (A) or unpaired conditioning (B and C). Activity for each single neuron was summed across the first 6 trials of Day 3 of training and normalized in 1-s bins to the mean baseline firing for each individual neuron. Filled arrowheads mark the time-point of shock-US delivery. Shock artifact prevented neuronal data from being collected during the presentation of the US. The CS-alone trials during unpaired training are shown in B. Prelimbic and infralimbic single neurons showed opposite patterns of activity in response to the CS and US. Prelimbic neurons showed increases in activity to the CS and US during trace conditioning but not during unpaired training. Infralimbic neurons showed decreases in activity to the CS and US during trace conditioning and little or no responding during unpaired training. Different scales were used on the ordinate axis of the prelimbic and infralimbic histograms because of the increasing and decreasing response patterns in these areas.

Prelimbic neurons exhibited a learning-related increase in activity during the 5-s CS, while infralimbic neurons showed a decrease in activity during the CS. The change in activity during the CS was converted to a z-score for each single neuron by comparing the activity during the 5-s CS to the average activity in baseline. Figure 3-4 shows the
mean change in prelimbic and infralimbic single neuron activity during the CS in 2-trial blocks across days of training. Figure 3-4A shows a greater increase in prelimbic single neuron activity during the CS across days of trace conditioning compared with unpaired training. A repeated measures ANOVA conducted on the data in Figure 3-4A showed a significant Training Group effect, $F(1,749) = 26.66, p = 0.00001$. Figure 3-4B shows that infralimbic neurons in the trace conditioning group exhibited a greater decrease in single neuron activity during the CS compared with the unpaired group. A repeated measures ANOVA conducted on the data in Figure 3-4B showed a significant Training Group effect, $F(1,355) = 20.95, p = 0.000007$.

![Figure 3-4: Inverse patterns of prelimbic and infralimbic responding to the tone-CS.](image)

Graphs show the mean change in single neuron activity from baseline during the 5-s tone-CS for single neurons recorded from the prelimbic (panel A) and the infralimbic (panel B) areas of the mPFC during trace or unpaired conditioning. Each point represents the mean standardized change in activity during the CS in a 2-trial block on each day of acquisition (Days 1-3) and on CS-alone retention days (Ret 1-2). Error bars = S.E.M. Arrowhead on the ordinate axis shows the level of no change in activity.

The mPFC responses to the US were similar in direction to the responses to the CS. Prelimbic neurons exhibited a learning-related increase in activity following the US, while infralimbic neurons showed a decrease in activity following the US. Figure 3-5A
shows the mean change in prelimbic single neuron activity following the US. This figure shows that prelimbic single neurons exhibited a greater increase in activity to the US during trace conditioning compared with unpaired training. A repeated measures ANOVA conducted on these data showed a significant Training Group X Day X Block effect, $F(4,870) = 3.22, p = 0.0123$. Follow-up analyses showed a significant Training Group difference on Block 2 of Day 1, Blocks 1 and 2 of Day 2, and Block 1 of Day 3. Figure 3-5B shows that infralimbic neurons in the trace conditioning group exhibited a greater decrease in single neuron activity following the US compared with the unpaired group. A repeated measures ANOVA conducted on these data showed a significant Training Group effect, $F(1,200) = 7.91, p = 0.0054$.

![Figure 3-5: Inverse patterns of prelimbic and infralimbic responding to the shock-US.](image)

Graphs show the mean change in single neuron activity from baseline during a 5-s response period following the shock-US for prelimbic (panel A) and infralimbic (panel B) single neurons recorded during trace or unpaired conditioning. The 5-s response period began 5 s after US offset. Each point represents the mean standardized change in activity to the US in 2-trial blocks on each day of acquisition (Days 1-3). Error bars = S.E.M. Arrowhead on the ordinate axis shows the level of no change in activity.
3.3.4 Prelimbic response profiles to the CS

The prelimbic neurons recorded during the three days of trace conditioning exhibited a greater percentage (57.9%; 102/176) of changes in responding to the CS (i.e., increases or decreases from baseline) compared with the neurons recorded during unpaired training (43.0%; 116/270; $z$-test of independent proportions = 3.096, $p = 0.002$). Overall, four distinct patterns of responding to the CS were observed for the prelimbic neurons recorded during trace and unpaired conditioning. Figure 3-6 shows the average normalized perievent histograms for these four types of prelimbic response profiles to the CS and the percentage of neurons that contributed to each profile. Figure 3-6A shows the most common prelimbic profile for the trace conditioning group, which was an increase in firing at CS onset (standard score >1.60) that persisted above baseline firing levels throughout the 5-s CS. The second prelimbic profile exhibited a brief increase in firing that lasted for only the first 1 s of the CS (Figure 3-6B), while a third profile showed increases in firing with an onset that occurred during the later half of the CS or began at CS-offset (Figure 3-6C). It is important to note that the neuron profiles in panels A and C showed on average elevated activity throughout much of the trace interval. Sustained responses during the trace interval were analyzed in greater detail in Figures 3-9 through 3-11. The three profiles of increased responding to the CS in panels A-C constitute 45.4% (80/176) of all recorded prelimbic single neurons during trace fear conditioning. A fourth subset of prelimbic neurons exhibited a decrease in firing during the 5-s CS (Fig. 3-6D). Figure 3-6 shows that prelimbic single neurons in the unpaired group exhibited response profiles to the CS that were similar in shape to the trace group. The
trace group, however, showed a greater percentage of neurons in the response profile shown in panel A (z-test of independent proportions = 3.193, $p = 0.0014$). Furthermore, a repeated measures ANOVA also revealed a significant Training Group X Day X Block interaction, $F(8,230) = 2.088$, $p = .0379$ for the standardized change in single neuron activity during the CS for the response profile in panel A. Follow-up analyses showed that there was a greater amount of firing in the trace group on Block 2 of Day 1 and Block 3 of Day 3. Analyses conducted on the remaining three profiles (panels B, C, and D) revealed no significant differences between groups. All four profiles were also observed on the two days of CS-alone trials following trace or unpaired conditioning (data not shown). The percentage of neurons in each profile shown in Figure 3-6 did not change significantly across days of training. Figure 3-7 shows the activity of exemplar prelimbic single neurons from each of the response profiles shown in Figure 3-6.
Figure 3-6: Prelimbic single neuron response profiles to the tone-CS.

Histograms show the average pattern of activity of all neurons in each of the four prelimbic response subclasses. Activity for each single neuron was summed across the first 6 trials and normalized in 1-s bins to the mean baseline firing for each individual neuron. Histograms were then formed by averaging the normalized histograms across Days 1-3 of trace or unpaired training. Filled arrowheads mark the time-point of the delivery of the shock-USs on 20-s trace conditioning trials. The majority of prelimbic neurons recorded during trace or unpaired training exhibited one of four patterns of activity to the CS: A. Increased firing during the entire 5-s CS; B. Increased firing only during the first 1 s of the CS; C. Increased firing only during the second half of the CS or immediately after CS offset; D. Decreased firing during the 5-s CS. Percentages represent the proportion of prelimbic single neurons recorded in each subclass during trace or unpaired training. The remaining proportion of prelimbic neurons (not shown) did not respond to the CS during trace or unpaired training.
Figure 3-7: Exemplar prelimbic single neurons from each of the four response profiles.

Each histogram shows the number of spikes/500-ms bin, summed across trials 1-6 for an example single neuron recorded during trace or unpaired training. Dot rasters above each histogram show action potential events on each of the first 6 trials (first trial on bottom). The inset in each histogram shows overlays of action potential waveforms for each example single neuron across the first two trials (vertical bar = 0.1 mV; horizontal bar = 0.5 ms).  

A. Example prefrontal single neurons that showed an increase in responding during the entire 5-s CS.  
B. Example prefrontal single neurons that showed a brief excitatory response to the CS only during the first 1 s after CS-onset.  
C. Example prefrontal single neurons that showed an increase in firing at the end of the CS.  
D. Example prefrontal single neurons that showed a decrease in firing during the CS.  

Solid arrowheads mark the latency of US delivery on 20-s trace conditioning trials. No USs were delivered on CS-alone retention trials following trace fear conditioning, but the open arrowhead marks the latency of US delivery on previous training trials.
3.3.5 *Infralimbic response profiles to the CS*

Infralimbic neurons showed only general increases or decreases in activity to the CS. Figure 3-8A shows the average normalized perievent time histograms of the increasing and decreasing response profiles averaged across days 1-3 of trace or unpaired conditioning. A greater percentage of infralimbic neurons recorded during trace conditioning showed a decrease (31.9%; 29/91) in responding to the CS as opposed to an increase (15.4%; 14/91) in responding to the CS ($z$-test of independent proportions = 2.62, $p = 0.0089$). In contrast, a greater percentage of infralimbic neurons recorded during unpaired training showed an increase (30.3%; 37/122) in responding to the CS rather than a decrease (12.3%; 15/122) in responding to the CS ($z$-test of independent proportions = 3.44, $p = 0.0006$). Figure 3-8B shows examples of single neurons from each response profile depicted in Figure 3-8A.
Figure 3-8: **Infralimbic response profiles to the tone-CS.**

**A. Average infralimbic responses**

- Histograms show the average pattern of activity of all neurons in each of the two infralimbic response subclasses. Activity for each single neuron was summed across the first 6 trials and normalized in 1-s bins to the mean baseline firing for each individual neuron. Histograms were then formed by averaging the normalized histograms across Days 1-3 of trace or unpaired training. Filled arrowheads mark the time-point of shock-US delivery on 20-s trace conditioning trials. Infralimbic response profiles obtained during trace or unpaired conditioning exhibited either an increase or decrease in firing during the CS. Percentages represent the proportion of infralimbic single neurons recorded in each subclass during trace or unpaired training. The remaining proportion of infralimbic neurons (not shown) did not respond to the CS during trace or unpaired training.

**B. Infralimbic single neurons**

- Histograms show exemplar individual single neurons obtained from each infralimbic response profile during trace or unpaired training. Each histogram shows the number of spikes/500-ms bin, summed across trials 1-6. Solid arrowheads mark the latency of US delivery on acquisition trials. A 20-s trace interval separated the CS offset and US on paired trials. Dot rasters above each histogram show action potential events on each of the first 6 trials (first trial on bottom). The inset in each histogram shows overlays of action potential waveforms for each example single neuron across the first two trials (vertical bar = 0.1 mV; horizontal bar = 0.5 ms).

### 3.3.6 Prelimbic sustained activity during the trace interval

Figures 3-3 and 3-6 demonstrate that some of the prelimbic neurons exhibited sustained increases in activity during a large part of the trace interval. These sustaining neurons may provide a bridging signal that allows for a Hebbian overlap of CS and US.
information during trace conditioning. Initial analyses showed that a very small percentage of single neurons in the paired group (1.3%; 4/302, across all 5 days) and no single neurons in the unpaired group (0/459, across all 5 days) exhibited increases in activity above baseline in all 20 1-s bins of the trace interval. Further analyses showed that many prelimbic single neurons exhibited increased activity during the trace interval with brief periods of inactivity in a few 1-s bins. Therefore, individual prelimbic neurons were classified as sustaining neurons if their activity increased above baseline for any 13 or more of the 20 1-s bins during the trace interval (i.e., at least 2/3 of the trace interval). A z-score computed the change in activity from baseline for each 1-s bin in the trace interval for each sustaining single neuron. These z-scores were averaged across the 6 trials of each of day of acquisition. The majority of all single neurons classified as sustaining neurons showed increased activity above baseline in the last 1-s bin in the trace interval (29/45 trace, 64.4%; 38/52 unpaired, 73.1%). This suggests that most of the sustaining neurons maintained an elevated activity pattern for the duration of the trace interval despite the brief 1-s periods during the trace interval at or below baseline levels of activity. Furthermore, analyses conducted on the overall average levels of activity for the sustaining neurons showed that the change in activity for each group during the last 1-s bin of the trace interval (i.e., bin 20) was significantly greater than the average baseline activity, \( t(44) = 3.47, p = 0.0012 \) for trace \( t(51) = 5.02, p = 0.00001 \) for unpaired. The normalized change in activity during the last 2 s of the trace interval was also significantly greater than baseline firing for each group of sustaining neurons, \( t(44) = 4.30, p = 0.0001 \) for trace and \( t(51) = 7.34, p = 0.00001 \) for unpaired.
Figure 3-9 shows the average normalized perievent time histograms for prelimbic neurons that exhibit sustained activity following the CS during trace and unpaired training. These histograms suggest that a subset of prelimbic neurons exhibits elevated activity following the CS in both trace and unpaired training situations. Analyses comparing the percentage of sustaining neurons between groups approached but did not reach significance ($z$-test of independent proportions = 1.425; $p = 0.15$). This suggests that prelimbic mPFC neurons may provide some level of post-CS tonic bridging activity regardless of the associative nature of the CS. Figure 3-10A shows examples of single neurons recorded during trace and unpaired conditioning that exhibited sustained firing following the CS.

Figure 3-9: Average prelimbic sustained activity during the trace interval.  
A subset of prelimbic single neurons showed sustained increases in firing during the 20-s trace interval. Histograms show the mean firing pattern for this subset of prelimbic neurons summed across the first 6 trials on each day of trace (panel A) or unpaired training (panel B). Firing rates in each 1-s bin were normalized to the mean baseline firing for each individual neuron, and histograms were then formed by averaging across all sustaining neurons. Filled arrowheads mark the time-point of the delivery of the shock-USs on 20-s trace conditioning trials. No USs were delivered on CS-alone retention trials following trace fear conditioning, but the open arrowhead marks the latency of US delivery on previous training trials. Percentages represent the proportion of total prelimbic neurons in trace or unpaired training that showed sustained activity during the trace interval on each day of training.
Figure 3-10: *Exemplar mPFC single neurons showing sustained firing during the trace interval.*

A. Each histogram shows the number of spikes/500-ms bin, summed across trials 1-5 for an example prelimbic sustaining single neuron recorded during trace (left) or unpaired training (right). Although there were no trace intervals in unpaired training, some neurons showed sustained responses on CS-alone trials. Sustained responses were larger in trace than in unpaired training. B. Two example infralimbic single neurons recorded during trace or unpaired conditioning showing moderate levels of sustained firing following CS-offset. Infralimbic sustained responses were much smaller than the prelimbic sustained responses. Solid arrowheads mark the latency of US delivery on trace conditioning trials. A 20-s trace interval separated the CS and US on paired trials. No USs were delivered on CS-alone retention trials following trace fear conditioning, but the open arrowhead marks the latency of US delivery on previous training trials. The inset in each histogram shows overlays of action potential waveforms for each example single neuron across the first two trials (vertical bar = 0.1 mV; horizontal bar = 0.5 ms).

Although prelimbic neurons exhibit sustained activity following the CS in both associative and non-associative situations, further analyses show that prelimbic neurons
exhibited a greater increase in firing during the CS and trace interval in the associative trace situation as compared with the non-associative unpaired situation. These analyses examined the amount of sustained activity during the CS and the amount of sustained activity during the subsequent trace interval. This was done by summing the normalized change in activity during the CS or the entire 20-s trace interval for each prelimbic sustaining neuron. Part A of Figure 3-11 shows that the sustaining neurons in the trace conditioning group had a greater amount of activity during the 5-s CS compared with the unpaired group. An ANOVA conducted on these CS data showed that there was a significant Training Group effect, $F(1,152) = 18.06, p = 0.000037$. Part B of Figure 3-11 shows that the sustaining prelimbic neurons from the trace group also showed a greater amount of activity during the 20-s trace interval compared with the unpaired group. The difference in Panel B was confirmed by a Training Group effect, $F(1, 152) = 5.37, p = 0.022$. An additional analysis compared the amount of activity during the 1st and 2nd 10-s periods of the trace interval. This analysis used Training Group and Day as between factors and trace interval Period as a repeated measure. The analysis revealed a significant Training Group X Period interaction, $F(1,152) = 8.10, p = 0.005$. Follow-up analyses showed that the trace conditioning group exhibited greater sustained activity during the 1st 10 s of the trace interval compared with the 2nd 10 s. The trace group also had greater sustained activity in the 1st 10 s of the trace interval compared with the unpaired group. The unpaired group showed no change in sustained activity from the 1st to 2nd half of the trace interval. Part C of Figure 3-11 shows the amount of combined activity during the CS and 20-s trace interval for the sustaining prelimbic single neurons. The data in Panel C also showed a significant Training Group effect, $F(1, 152) = 13.36, p$
= 0.00035. Together, the data in Figure 3-11 show that there is a greater amount of sustained activity during the trace interval in the associative trace situation compared with the non-associative unpaired situation, and this learning-related sustained activity during the trace interval may be due in part to associative increases in activity that begin during the presentation of the CS. This is consistent with the data in Figures 3-3 and 3-4 that show that there is a greater amount of prelimbic responding during the CS in the trace situation compared with the unpaired situation. Moreover, analyses revealed that there was a greater percentage of the sustaining prelimbic neurons in the trace group (62.2%; 46/74, across all 5 days) that showed increases in activity specifically during the CS compared with the unpaired group (42.0%; 37/88, across all 5 days), \( z \)-test of independent proportions = 2.552, \( p = 0.0107 \).
Figure 3-11: *Amount of normalized firing during the CS and trace interval for prelimbic neurons showing sustained activity.*

Graphs show the mean normalized activity during the CS and/or trace interval for the prelimbic single neurons that exhibited sustained increases in firing during the trace interval. There was a greater amount of activity during the CS and trace interval for the sustaining prelimbic neurons recorded from the trace animals compared with the unpaired animals. Firing was normalized to baseline for each single neuron. Amounts of firing were then calculated for each single neuron by summing the firing in each 1-s bin during the 5-s CS or 20-s trace interval. Each point represents the mean firing in a 6-trial block on each day of training (Days 1-3) and on CS-alone retention days (Ret 1-2). The mean activity is shown during the 5-s CS (A); the 20-s trace interval (B); and the 25-s period including the CS and trace interval (C). Error bars = S.E.M.

Similar criteria for sustained trace interval activity were applied to the infralimbic neurons. Although a modest proportion of infralimbic neurons in the trace (20.4%; 37/181) and unpaired groups (19.6%; 38/194) were classified as sustaining neurons using these criteria, the overall pattern of sustaining activity for the infralimbic neurons was poor. Two of the better individual examples of sustaining infralimbic neurons are shown
in Figure 3-10B. These individual examples and the average patterns of sustaining infralimbic neurons (not shown) show only small increases in activity during the trace interval. An ANOVA was conducted to determine if there was less sustaining activity during the trace interval in the infralimbic neurons compared with the prelimbic neurons. This ANOVA included the factors Site (prelimbic vs. infralimbic), Training Group, and Days. The analysis revealed significant effects of Training Group, $F(1,218) = 5.46, p = 0.020$, and Site, $F(1,218) = 4.62, p = 0.033$. This suggests that the prelimbic area of mPFC plays a larger role in sustaining the neural representation of the CS across the trace interval compared with the infralimbic area of mPFC.

### 3.4 Discussion

This study recorded single neuron activity from the prelimbic and infralimbic areas of the rat mPFC during trace fear conditioning or unpaired control training. Prelimbic neurons exhibited learning-related increases in activity to the CS and US during trace fear conditioning. A subset of prelimbic neurons also showed sustained increases in activity during the trace interval separating the CS and US. Infralimbic neurons exhibited learning-related decreases in activity to the CS and US, but did not exhibit the level of sustained trace interval activity that was observed in the prelimbic area. These results suggest that the prelimbic and infralimbic areas of mPFC have unique roles in processing trace fear stimuli, and that single neurons in the prelimbic area provide sustained activity that may bridge the trace interval separating the CS and US during trace fear conditioning.
The sustained single neuron responses observed in the prelimbic area may provide a bridging pattern of activity that allows for a Hebbian-like overlap of CS and US information during trace fear conditioning. Hebbian patterns of overlapping activity are an integral part of computational learning models that involve temporal intervals (e.g., Mongillo, Amit, & Brunel, 2003; Reutimann, Yakovlev, Fusi, & Senn, 2004; Rodriguez & Levy, 2001; Wallenstein, Eichenbaum, & Hasselmo, 1998). A number of neurophysiological studies have shown that mPFC neurons exhibit sustained patterns of activity during temporal intervals. Tonic increases in mPFC activity have been shown during the delay period in delayed matching-to-sample tasks in rats (J. Y. Chang, Chen, Luo, Shi, & Woodward, 2002). Similar tonic patterns of activity have been shown during matching tasks in the dorsolateral prefrontal cortex of primates (Funahashi, Bruce, & Goldman-Rakic, 1989; Fuster, 1973; Sawaguchi & Yamane, 1999), a site that may be analogous to the mPFC in rats (Conde, Maire-Lepoivre, Audinat, & Crepel, 1995). In other work, Fuster and colleagues showed that monkey prefrontal neurons exhibit sustained activity during a 10-s delay period separating an auditory stimulus and an appropriate color stimulus (Fuster, Bodner, & Kroger, 2000).

The majority of the prelimbic neurons that were classified as sustaining neurons showed increases in activity above baseline for 13 to 15 s of the 20-s trace interval (71.1%; 69/97, trace and unpaired groups across the first 3 days). These periods of increased trace interval activity were interrupted by a few 1-s bins when the neuron fired at or below baseline levels; nevertheless, these prelimbic sustaining neurons exhibited a persistent pattern of increased firing throughout the trace interval. Moreover, despite this intermittent pattern of elevated trace interval activity, most of the sustaining single
neurons showed increased firing in the final 1-s bin of the 20-s trace interval (69.1%, 67/97 in both groups). This may suggest that prelimbic single neurons provide a relatively sparse firing pattern that tonically bridges the entire 20-s duration of the trace interval separating the CS and US.

There are some indications in the present study that prelimbic neurons may bridge the duration of the trace interval cooperatively, such that each individual prelimbic neuron does not have to fire continuously for the entire 20-s trace interval and hence carry the entire code of the trace interval duration. There is evidence for this in Figure 3-9 which shows that the population of sustaining neurons on average contributes increases in activity above baseline for the entire trace interval. Furthermore, Figure 3-6 describes several distinct subclasses of prelimbic neurons, and together these subclasses may contribute to the sustaining activity code. For example, one subclass may contribute to sustaining activity that begins at CS onset and another that begins at CS offset (i.e., Classes A and C). The other subclasses of prelimbic neurons may play a role in signaling the onset of the CS (i.e., Class B) or may be part of an inhibitory network within the prelimbic area (i.e., Class D).

Interestingly, prelimbic neurons exhibited sustained increases in activity following the CS in both the trace and the unpaired control situations. The sustained increases in prelimbic activity in the unpaired control situation were smaller in magnitude than in trace conditioning; however, the percentage of prelimbic single neurons exhibiting sustained increases in activity was similar between the trace and unpaired training situations. This may suggest there is a general role for prelimbic neurons to sustain activity following the CS regardless of the associative nature of the stimulus.
Moreover, prelimbic neurons may initially exhibit sustained activity in response to salient stimuli, but only in associative situations do these sustained responses become strengthened as associative training continues. The results in this chapter support this idea because trace and unpaired groups had similar amounts of sustained activity on day 1 of training, but the amount of sustained activity increased slightly across days of trace fear conditioning but not across days of unpaired training. Similar findings were obtained in a trace eyeblink conditioning study that recorded single neurons from the anterior cingulate cortex, a site that is adjacent to the mPFC (Weible, Weiss, & Disterhoft, 2003). The Weible study found that the paired and unpaired groups exhibited increases in anterior cingulate activity during the CS/trace-interval early in training, but only the paired group maintained these increases over subsequent days of conditioning.

It is important to note that the configuration of single neurons on each electrode changed slightly from one day of training to the next in our study. This raises the possibility that some of the changes in activity across days of training may be the result of changes in the configuration of neurons on each electrode. This possibility is highly unlikely considering the consistent patterns of neuronal activity across days of training for each individual training group, and the relatively large number of neurons that were sampled from each training group.

Four profiles of prelimbic single neuron responding to the CS were identified in this study. Only one of the four response profiles (i.e., the profile that responded for the entire 5-s CS in Figure 3-6A) revealed a greater percentage of neurons in the paired versus the unpaired group. This was also the only response profile that showed a greater amount of action potential firing during the CS in trace rather than unpaired training.
This suggests that a very specific subset of neurons in mPFC has an activity pattern that is unique to the CS-US association. The three remaining classes of prelimbic single neuron response profiles showed no quantitative differences between the trace and unpaired training situations. It is possible that these three remaining single neuron response profiles do not have a direct associative role in trace fear conditioning. Alternatively, it is also possible that the three remaining response profiles do not distinguish between the trace and unpaired training situations because of contextual similarities between the associative and non-associative situations. Moreover, both the paired and unpaired training situations present the CS within the same conditioning context, and rats may associate the context with the US and further associate the CS with the context through a second-order conditioning process. Others have suggested that this second-order conditioning process may occur during backward and unpaired conditioning procedures (R. C. Chang, Stout, & Miller, 2004; Droungas & LoLordo, 1994). Thus, some of the mPFC single neuron responses to the tone-CS may represent encoding of the CS-context relationship, and this could explain some of the similarities in CS responding between the trace and unpaired situations.

Infralimbic single neurons exhibited learning-related decreases in activity to the CS and US, a pattern that was opposite to the prelimbic region. These inverse patterns of prelimbic and infralimbic activity are consistent with a number of studies which suggest that these areas have unique roles in learning processes. Studies show that lesions restricted to the prelimbic area disrupt the acquisition of delayed alternation tasks and delayed matching-to-sample tasks (Delatour & Gisquet-Verrier, 2001; Granon, Vidal, Thinus-Blanc, Changeux, & Poucet, 1994). In contrast, lesions of the infralimbic area
have been shown to have little impact on the acquisition of conditioned fear responses, but these same lesions are able to disrupt the extinction of fear responses (Morgan, Romanski, & LeDoux, 1993). This suggests that the infralimbic area plays a role in inhibiting responses to non-reinforced stimuli. Another study supports this notion by showing that lesions in the infralimbic region of the mPFC impairs the ability of a rat to learn to inhibit stepping down to an area where it was previously shocked (Jinks & McGregor, 1997).

Anatomical data provide further support for the unique roles of the prelimbic and infralimbic areas in learning. The majority of projections from the prelimbic area terminate in the nucleus accumbens, basal lateral amygdala, medial dorsal thalamus, and ventral tegmental area (e.g., Vertes, 2004); whereas the infralimbic area projects to the lateral hypothalamus, periaqueductal grey, parabrachial nucleus, the nucleus of the solitary tract, and the amygdala (Fisk & Wyss, 2000; McDonald, Mascagni, & Guo, 1996; Vertes, 2004).

Several studies suggest that the infralimbic region of mPFC has a unique role in modulating the activity of the amygdala during the extinction of fear responses (e.g., Quirk, Likhtik, Pelletier, & Pare, 2003). Milad and Quirk (2002) showed that on CS-alone extinction trials following delay fear conditioning, single neurons in the infralimbic but not the prelimbic region exhibited increased firing at CS onset. Their study also showed that stimulation of the infralimbic area at CS onset on extinction trials accelerated the extinction of learned fear responses. This suggests that excitatory infralimbic activity during extinction inhibits the expression of fear responses when these responses are no longer appropriate. Some of the infralimbic single neuron responses
from the present study are consistent with this notion. Infrahlimbic neurons showed a small increase in firing to the CS and US during non-reinforced unpaired training, and a decrease in firing to the CS and US during paired trace fear conditioning. In contrast to the findings of Milad and Quirk (2002), however, infralimbic neurons in the present study did not show substantial increases in activity during the CS-alone extinction trials that followed trace fear conditioning. This was an unexpected finding which was probably due to the relatively small number of CS-alone extinction trials that were presented in our study which did not allow for the complete extinction of conditioned HR responses.

This chapter describes tonic and phasic neuronal activity patterns in mPFC that were specific to the CS-US association in trace fear conditioning. These findings were obtained by comparing single neuron responses during trace and unpaired control conditioning. It is unclear from our study if these types of mPFC single neuron responses occur when longer or shorter trace interval durations are used, or when no trace interval separates the CS and US (i.e., delay fear conditioning). Baeg et al. (2001) recorded mPFC single neurons during CS-alone retention trials following delay fear conditioning or following trace fear conditioning with a 2-s trace interval. They found similar CS response profiles in the trace and delay fear conditioning groups, and both of these groups had greater proportions of CS-responsive neurons compared with naïve control animals. Based on the many similarities in responding between the trace and unpaired groups in the present study, it is entirely possible that the neuronal response profiles are also very similar between fear conditioning paradigms with short and long duration trace intervals.
Chapter 2 reported that single neurons in the DG and CA1 sub-regions of the hippocampus exhibit learning-related patterns of activity to the CS and US during trace fear conditioning, but neurons in these areas of the hippocampus did not exhibit sustained increases in firing that bridged the trace interval (Gilmartin & McEchron, 2005a). The present study showed that a subset of neurons in mPFC exhibit increases in activity that are sustained throughout most of the trace interval. The hippocampus and mPFC are both necessary for trace fear conditioning (Chowdhury, Quinn, & Fanselow, 2005; McEchron, Bouwmeester, Tseng, Weiss, & Disterhoft, 1998; Quinn, Oommen, Morrison, & Fanselow, 2002; Runyan & Dash, 2004; Runyan, Moore, & Dash, 2004), and the hippocampus sends strong projections to the mPFC (Chiba, 2000; Ishikawa & Nakamura, 2003, 2006; Jay & Witter, 1991). Thus, it is likely that the hippocampus and mPFC form a critical part of the trace fear conditioning network where learning-related information about the CS and US are transferred from the hippocampus to mPFC so that Hebbian-like associations can take place. Connections between mPFC and the amygdala may then provide a pathway for the expression of trace fear conditioned responses (Conde, Maire-Lepoivre, Audinat, & Crepel, 1995; Ishikawa & Nakamura, 2003; Vertes, 2004).

Clearly, this is a very simplified model, and more than likely there are other intricacies to the network that account for the multiple phases of trace fear conditioning (e.g., acquisition, retention, and extinction). Nevertheless, this trace fear conditioning network model may provide a useful tool for understanding the role of the hippocampal-prefrontal system in learning and memory. Chapter 4 will examine activity in the hippocampus and mPFC simultaneously during trace fear conditioning to determine whether these two structures interact during the learning process. Specifically, Chapter 4 will record the
field activity of neuronal populations in each structure to test the hypothesis that rhythmic population activity becomes synchronized in the hippocampus and mPFC during the trace interval. Other work provides support for a hippocampal-prefrontal interaction in trace fear conditioning. Knight et al. (2004) showed that during trace fear conditioning in humans frontal cortical regions were activated during the trace interval, while the hippocampus was activated during the CS and US but not during the trace interval. Lee and Kesner (2003) showed that the hippocampal-prefrontal system is necessary for performing a radial arm maze spatial memory task in which temporal intervals separate sample and choice trials. Several recent studies on spatial working memory showed that many single neurons in the rat mPFC were entrained to hippocampal theta oscillations specifically when they exhibited behavior-related activity (Hyman, Zilli, Paley, & Hasselmo, 2005; Jones & Wilson, 2005b; Siapas, Lubenov, & Wilson, 2005).

The results of this chapter show that prelimbic and infralimbic neurons have unique roles in processing learning-related information during trace fear conditioning. This study also provides evidence that the mPFC is an important part of the trace fear conditioning network where sustained patterns of activity during the trace interval can allow Hebbian-like associations of CS and US information. The results of Chapters 2 and 3 suggest that the hippocampus and mPFC interact to encode the CS-trace-US association. Chapter 4 will examine field activity in the hippocampus and mPFC to determine whether these two sites interact to encode the trace interval.
Chapter 4

Coordination of Rhythmic Activity in the Medial Prefrontal Cortex, Hippocampus, and Amygdala during Trace Fear Classical Conditioning

4.1 Introduction

Chapters 2 and 3 showed that the hippocampus and medial prefrontal cortex (mPFC) are key sites in the trace fear network that encode unique components of the trace fear conditioning trial. Chapter 2 showed that single neurons in the CA1 and DG regions of the hippocampal formation exhibit learning-related changes in activity during the CS and US periods, but do not exhibit learning-related patterns of activity during the trace interval (Gilmartin & McEchron, 2005a). Chapter 3 found that a subset of the prelimbic single neurons in the mPFC exhibit tonic increases in activity during the empty trace interval that may serve to bridge the gap between the CS and US in trace conditioning (Gilmartin & McEchron, 2005b). Together, Chapters 2 and 3 suggest that the hippocampus and mPFC work together during trace fear conditioning to encode the trace interval. There is some evidence for this from other studies showing synchronized rhythmic field activity in the hippocampus and mPFC during specific phases of spatial working memory tasks (e.g., Jones & Wilson, 2005b). This chapter sought to determine if activity in the hippocampus and mPFC is coordinated during trace fear conditioning. This chapter will also examine activity in another major structure in the fear conditioning network, the amygdala. This will determine if the hippocampus or mPFC show
coordinated activity with the major behavioral output structure in fear conditioning. Recent support for coordination between the hippocampus and amygdala comes from studies by Pape and colleagues, which showed coordinated hippocampal and amygdalar activity during the retention of fear memories (Pape, Narayanan, Smid, Stork, & Seidenbecher, 2005; Seidenbecher, Laxmi, Stork, & Pape, 2003).

Electroencephalographic (EEG) field activity recordings were used in this chapter to examine coordinated activity in the three major sites of the trace fear conditioning network, the hippocampus, amygdala, and mPFC. Coherence analyses were applied to rhythmic field activity recorded simultaneously from each site to determine if oscillatory activity in the hippocampus, amygdala, and mPFC becomes synchronized during different phases of the learning process. This chapter focuses on coordinated activity around the theta frequency range (2-8 Hz), because rhythmic activity in this range has been shown to be important for learning and memory processes in animals (Berry & Thompson, 1978; B. Givens, 1996; Griffin, Asaka, Darling, & Berry, 2004; Pare, Collins, & Pelletier, 2002), including humans (Jacobs, Hwang, Curran, & Kahana, 2006; Jensen & Tesche, 2002; Raghavachari et al., 2001; Raghavachari et al., 2006). Furthermore, other studies have shown coordinated activity between the hippocampus, mPFC, and amygdala at theta frequencies (Hyman, Zilli, Paley, & Hasselmo, 2005; Jones & Wilson, 2005b; Pape, Narayanan, Smid, Stork, & Seidenbecher, 2005; Seidenbecher, Laxmi, Stork, & Pape, 2003; Siapas, Lubenov, & Wilson, 2005). We hypothesize that the hippocampus, mPFC, and amygdala exhibit coordinated theta activity during specific phases of trace fear conditioning.
4.2 Materials and Methods

4.2.1 Subjects and surgery

Surgery was performed on a total of 37 adult male Sprague-Dawley rats in this study (200-400 g; Harlan, IN). Nine of these 37 rats were excluded from analyses because of improper electrode placement in 2 or more sites. Thus, group numbers include only these 28 rats. All rats were housed individually and received food and water ad libitum. All surgical procedures were in accordance with the National Institutes of Health and the Pennsylvania State University Institutional Animal Care and Use Committee. Rats were anesthetized with 1-2% halothane in 100% O\textsubscript{2} during surgery. Eyes were kept moist with antibacterial ophthalmic ointment. Each rat was positioned in a stereotaxic frame and the skull was exposed. Four self-tapping screws (#0-4.75mm, Small Parts, Inc., FL) were inserted ~1 mm into the skull to anchor the final dental cement head assembly. Three ipsilateral 3-mm diameter holes were drilled in the skull and 1-2 Teflon-coated stainless steel EEG recording electrodes (50 μm diameter each; tip separation: 200-400 μm) were stereotaxically lowered into the left prelimbic area of the medial prefrontal cortex (2.7 mm anterior to Bregma, 0.7 mm lateral to the midline, 3.2-3.5 mm ventral to the dura), left dorsal CA1 hippocampus (5.6 mm posterior to Bregma, 5.0 mm lateral to the midline, 2.1-2.4 mm ventral to the dura), and left basolateral amygdala (3.3 mm posterior to Bregma, 5.2 mm lateral to the midline, 7.0-7.6 mm ventral to the dura). Neuronal activity was monitored from one electrode in each site while the electrodes were being lowered in order to achieve optimal placement. Dental
cement was used to secure the electrodes to the skull and close the remaining wound area. Skull screws were used as the ground and reference electrode.

4.2.2 Paired and unpaired fear conditioning

Prior to training, the rats received two consecutive days of acclimation to the conditioning chamber (15 minutes/day). No stimuli were presented during the acclimation sessions. During acclimation, training, and testing sessions, the rats were allowed to move freely within the conditioning chamber by means of a commutator connecting the recording headstage with the amplifiers. All sessions took place within a sound-attenuating chamber. Electrocardiographic (EKG) activity was recorded across two leads (stainless steel wound clips) placed on the chest. Increases in heart rate were used to indicate learning in this study. EKG recordings allowed us to measure changes in heart rate across each trial. The EKG activity was amplified 10,000X, filtered between 10 and 1000 Hz, and sampled at 25 kHz. The auditory tone-CS (5 s; 6000 Hz; 80 dB) was delivered through a speaker at one end of the chamber. The shock-US (0.8 s; 1.0-mA scrambled, alternating current) was delivered through the floor of the conditioning chamber.

One day after the final acclimation session, each rat received three consecutive days of trace fear conditioning (Trace group, n = 17) or unpaired training (Unpaired group, n = 11). All rats then received one session of CS-alone retention trials 24 hours later.
A total of 17 rats received three consecutive days of 20-s trace fear conditioning. Each 20-s trace fear conditioning session consisted of 6-8 paired CS-US trials using an intertrial interval (ITI) of 200 ± 20 s, in which the offset of the tone-CS was separated from the onset of the shock-US by an empty 20-s trace interval on each trial. In some cases, rats exhibited excessive movement after 6 or 7 CS-trace-US trials. For these cases training was halted on this day, and additional trials were delivered on the following day of training. Twenty-four hours after the third trace fear conditioning session, rats received 10 CS-alone retention trials.

A total of 11 rats received three consecutive days of unpaired pseudoconditioning trials. The unpaired sessions were used to determine the non-associative level of behavioral and neuronal responding. Each unpaired session consisted of 6-8 CS-alone trials and 6-8 US-alone trials (ITI = 100 ± 20 s). During each unpaired session, the same stimulus was never presented more than two consecutive times. One day after the last unpaired session, rats received 10 CS-alone trials.

4.2.3 Electroencephalographic field activity recording

Electroencephalographic (EEG) analog signals were amplified (10,000X), filtered (1 Hz to 1 kHz), and collected with a DT-3100 Data Translation board (Data Translation, Marlboro, MA) attached to an 1.5-GHz computer, which sampled each channel at 25 kHz. The same computer sampled EKG activity and controlled the delivery of all stimuli. EEG and EKG data were collected for each 57-s trial starting from 16 s before CS onset to 16 s after US onset using software developed in our laboratory.
4.2.4 Analyses

All computational and statistical analyses were performed with the aid of Microsoft Visual Basic version 6.0 (Redmond, WA) and Matlab version 7.2 (The MathWorks, Inc.; Natick, MA) routines developed in our laboratory, Minitab Statistical Software version 10.0 (State College, PA), and Statistica software version 6.0 (Tulsa, OK). EEG signals were downsampled to 1000 Hz prior to analysis. Artifact in each signal was identified using a threshold-crossing criterion. Deflections in the signal that were greater than 5 standard deviations of the mean signal in baseline were marked as artifact. Bins containing artifacts were omitted from all analyses.

Power spectra for each EEG signal were calculated in 1 s or 5 s periods in each trial using the Welch method of a short-time fast Fourier transform and a Hanning window of 1 s (Welch, 1967). Power spectra were calculated in 1 s windows for the construction of dynamic spectra color plots (Figure 4-3). Relative power in the theta frequency band was obtained by normalizing the integral power between 2 and 8 Hz to the total power of the spectrum between 1 and 50 Hz. Group comparisons of relative theta power in percent change from baseline were performed using factorial repeated-measures ANOVAs. Comparisons of mean relative power between periods within each group were performed using repeated-measures ANOVAs or paired t-tests. A Fisher’s Least Significant Difference post-hoc test was used to test the significance of mean differences. An α level of 0.05 was required for significance in all analyses.

Coherence estimates were obtained for each pair of EEG signals on each trial during trace and unpaired conditioning. Coherence measures the spectral covariance of
two signals, an indicator of how well two signals are correlated at each frequency. Coherence between two EEG signals was measured according to Equation 4.1, where the coherence of two signals, $x$ and $y$, is equal to the magnitude of the cross-spectrum of the two signals ($P_{xy}$) at each frequency $f$, normalized by the auto-spectra of each signal ($P_{xx}$ and $P_{yy}$, respectively). The resulting coherence estimate is a value between 0 and 1 where 0 represents no correlation and 1 represents perfectly correlated oscillations at a given frequency.

$$C_{xy}(f) = \frac{|P_{xy}(f)|^2}{P_{xx}(f) * P_{yy}(f)}$$

4.1

Coherent signals have a constant phase relationship, where a zero phase difference indicates the two signals are synchronous in time. The term synchronous is used in this chapter to indicate coherent signals with a constant, but not necessarily zero, phase difference. Spectral coherence estimates require the averaging of multiple windows within each analysis period. Coherence of EEG signals in this study was calculated in 5 s periods in each trial using the Welch method and overlapping 1024-ms hanning windows. Coherence analysis of field activity in any 2 sites included only those rats that showed reliable EEG field activity in both sites. Therefore, coherence of hippocampus-mPFC activity included 16 of 17 trace rats and 10 of 11 unpaired rats; hippocampus-amygdala included 13 of 17 trace rats and 8 of 11 unpaired rats; mPFC-amygdala included 11 of 17 trace rats and 7 of 11 unpaired rats. Group comparisons of coherence in percent change from baseline were performed using factorial repeated-measures ANOVAs. An $\alpha$ level of 0.05 was required for significance in all analyses.
An increase in HR was the conditioned response in this chapter. Software routines developed in our laboratory measured HR by calculating the time between the R-peaks in the raw EKG waveform and calculating beats per min in each second. Changes in HR were analyzed using repeated measures ANOVAs, with an $\alpha$ level of 0.05 required for significance. All HR records containing arrhythmias, high baseline variability, or excessive movement artifact were excluded from analyses. These criteria resulted in less than 6% of the total trials being excluded from the final analyses.

4.2.5 Histology

Marking lesions were placed at the tips of all electrodes by passing direct current (20 $\mu$A) for 15 s. Rats were deeply anesthetized with 5% halothane in 100% O$_2$, sacrificed by decapitation, and the brains were placed in a 10% formalin solution (0.9% saline). Brains were then frozen, sectioned coronally, mounted on glass slides, and stained with neutral red. A light microscope was used to locate electrode tips.

4.3 Results

4.3.1 Electrode placement

Neuronal and behavioral analyses were conducted on the 28 rats that showed reliable LFP recordings from at least 2 of the 3 targeted sites: the CA1 hippocampus, the
prelimbic area of the mPFC, and the basolateral amygdala. Figure 4-1 shows the location of the electrode tips placed correctly in all three areas for one of the rats in this study.

Figure 4-1: Electrode placement in hippocampus, prelimbic, and basolateral amygdala
Coronal diagrams show the locations of the electrode tips in the prelimbic area of the medial prefrontal cortex, the basolateral amygdala, and dorsal CA1 hippocampus. Insets show the neutral red stained coronal slices with marking lesions from an electrode in each site for a rat in the trace conditioning group. Diagrams are reprinted from The Rat Brain in Stereotaxic Coordinates, 4th ed., G. Paxinos and C. Watson, Copyright 1998, with permission from Elsevier.

4.3.2 Heart rate fear conditioning

Unlike previous chapters, animals in this chapter were freely moving during trace fear conditioning. As a result, increases in HR (i.e., tachycardia) were used to indicate
conditioned fear responses in this chapter. Previous chapters used decreases in HR (i.e., bradycardia) as the conditioned fear response in restrained rats. A recent study from our laboratory shows that restrained rats exhibit conditioned bradycardiac responses during the trace interval, but freely moving rats exhibit conditioned tachycardiac responses (McEchron, Alexander, & Gilmartin, in prep). Changes in HR from baseline were calculated in 1-s bins averaged across trials 2-6 of each session of training. Trial 1 was excluded from these analyses because a number of rats in the trace group exhibited high variability in baseline HR on this trial. This is likely a result of exploration in the freely moving situation before the first paired trial was delivered. Trials 2-6 therefore represent the block of trials that best characterizes fear responses in both groups. Figure 4-2 shows the mean change in HR during the middle 10 s of the trace interval across days for the trace and unpaired groups. Consistent with our other studies, the trace fear conditioning group showed conditioned fear responses during the trace interval by the third day of training, while the unpaired group showed no conditioned fear responses. This tachycardiac response diminished after several non-reinforced CS-alone trials on the retention day. Analyses of the HR changes in Figure 4-2 for the 3 days of acquisition revealed a significant Training Group X Day interaction, $F(2,52) = 3.83, p = 0.0282$. Follow-up analyses showed a significant difference between the trace and unpaired groups on day 3 of acquisition ($p < 0.05$). The group difference approached significance on day 2 ($p = 0.0788$). HR and coherence analyses in this study were conducted on the middle 10-s period of the trace interval in order to examine behavioral and neuronal activity that is specific to the trace interval rather than the 5-s transition period.
immediately following the CS. Additional HR and coherence analyses examined the last 15-s of the trace interval and showed similar results (data not shown).

Figure 4-2: Conditioned heart rate fear responses.
Graph shows the mean change in HR from baseline during the middle 10 s of the trace interval averaged across trials 2-6 on each day of conditioning (Days 1-3) and on the CS-alone retention session (Ret) following trace or unpaired conditioning. Trace conditioned animals exhibited a larger increase in HR during the trace interval compared with unpaired animals. Error bars = S.E.M.

4.3.3 Frequency profiles in the hippocampus, prelimbic, and basolateral amygdala

Field activity in each site showed rhythmic activity in the 2-8 Hz frequency range. This range corresponds to the theta rhythm (Vanderwolf, 1969), which is prominent in animals and humans during various forms of learning (Berry & Seager, 2001; Buzsaki, 2002, 2005; Kahana, Seelig, & Madsen, 2001; Pare, Collins, & Pelletier, 2002; Raghavachari et al., 2001; Sederberg, Kahana, Howard, Donner, & Madsen, 2003).

Figure 4-3 shows the dynamic slow-wave frequency spectrum of the field activity recorded from each site in an exemplar trace-conditioned animal. The power of each frequency in the EEG signal is plotted in color for each second in a 57 s trace conditioning trial. This figure shows the presence of strong oscillatory activity around
the theta range (2-8 Hz) during the CS and trace interval in the hippocampus, mPFC, and amygdala. The remainder of the analyses in this study focused on this 2-8 Hz theta range to determine if field activity is coordinated between sites during trace fear conditioning.

Figure 4-3: Dynamic spectral power of field activity in each site.
The color plots show the power spectral density of each EEG signal recorded from a rat on day 2 of trace fear conditioning. The spectra are plotted in 1-s bins of a single trial and the amount of power at each frequency is represented by the color gradient, where black equals 0 power. Power spectra are normalized to the maximum power in each signal prior to the US delivery. Grey boxes denote the presentation of the 5-s CS and arrowheads mark the point at which the US was delivered.

Group analyses were conducted by normalizing theta power with respect to the total power between 1 and 50 Hz for each signal. Changes in relative theta power from baseline were then calculated for each site during the CS. Figure 4-4 shows the mean percent change in theta power during the CS across days of training. This figure shows
that the trace and unpaired groups exhibited opposite changes in theta power during the CS. Repeated-measures ANOVAs using Training Group and Day factors were conducted on the percent change in CS theta power in each site across 4 days of training. For the hippocampus (Fig. 4-4A), this analysis revealed a Training Group effect, $F(1,25) = 5.57, p = 0.0167$. A similar Training Group effect was revealed in the mPFC (Fig. 4-4B), $F(1,24) = 13.19, p = 0.0013$. The change in CS theta power in the amygdala showed a Training Group effect that approached significance (Fig. 4-4C), $F(1,18) = 3.06, p = 0.0969$.

Figure 4-4: Change in theta power during the CS

Graphs show the mean change in relative theta power from baseline levels during the CS for each group across 3 days of trace fear conditioning or unpaired conditioning (Days 1-3) and the retention session (Ret). Each point represents the mean change in relative theta power averaged across 8 trials each day for the EEG signals in the hippocampus (A), mPFC (B), and amygdala (C). Error bars = S.E.M.

Further inspection of the data in Figure 4-4 shows that trace conditioned animals exhibited a moderate increase in relative theta power during the CS. A separate set of analyses were conducted on the two groups to determine whether the changes in CS theta power in Figure 4-4 were significantly greater than baseline. Repeated-measures ANOVAs using Training Group, Day, and Period (baseline and CS) factors were conducted on the relative theta power in each site. For the hippocampus, this analysis
revealed a Training Group X Day X Period interaction, $F(3,75) = 2.80$, $p = 0.0456$. Follow-up analyses showed that the trace group exhibited a significant increase in CS theta power compared with baseline on day 2 ($p < 0.05$) and that the unpaired group showed a significant decrease in CS theta power on days 1-3 ($p < 0.05$). Analysis of prefrontal theta power revealed a Training Group X Period interaction, $F(1,24) = 12.19$, $p = 0.0019$. Follow-up analyses showed that the unpaired group exhibited a significant decrease in CS theta power compared with baseline ($p < 0.05$). Analysis of amygdala theta power revealed a Day effect, $F(3,54) = 5.08$, $p = 0.0036$. Follow-up analyses on the main effect of Day showed that overall theta power was significantly greater on day 1 compared with days 2-4 for both groups. Part C of Figure 4-4 showed an increase in theta power during the CS for the trace group. This suggests that amygdalar theta power may exhibit CS-evoked changes early in training. An ANOVA using Training Group and Period factors was applied to the amygdalar theta power on day 1 and revealed a Training Group X Period interaction, $F(1,19) = 4.72$, $p = 0.0425$. Follow-up analyses confirmed that the trace group showed an increase in theta power during the CS compared with baseline ($p < 0.05$). Overall, these analyses show that the trace group exhibits an increase in hippocampal and amygdalar theta power during the CS early in training, while the unpaired group shows a decrease in hippocampal and prefrontal theta power across days.

Analyses also examined changes in theta power following the US. Figure 4-5 shows the mean percent change in theta power during the 10 s period following the US for each day of trace fear conditioning or unpaired control training. This figure shows that the trace and unpaired groups exhibited opposite changes in US theta power in the hippocampus, but these groups exhibited similar decreases in theta power in the mPFC
and amygdala. A repeated-measures ANOVA using Training Group and Day factors on the change in hippocampal theta power in Figure 4-5A revealed an effect of Training Group, $F(1,25) = 10.05, p = 0.0040$. The trace group did not show a difference in US theta power compared with the unpaired group in either the mPFC or amygdala.

![Figure 4-5: Change in theta power following the US](image)

Graphs show the mean change in relative 2-8 Hz power from baseline levels during the 10 s following the US for each group across 3 days of trace fear conditioning or unpaired conditioning (Days 1-3). Each point represents the mean change in relative theta power averaged across 8 trials each day for the EEG signals in the hippocampus (A), mPFC (B), and amygdala (C). Error bars = S.E.M.

Further inspection of the data in Figure 4-5 shows that trace conditioned animals exhibited a decrease in relative theta power following the US in the mPFC and amygdala. A separate set of analyses were conducted on the two groups to determine whether the changes in theta power following the US in Figure 4-5 were significantly different from baseline. Repeated-measures ANOVAs using Training Group, Day, and Period (baseline, US) factors were conducted on the relative theta power in each site. For the hippocampus, the analysis revealed a Training Group X Period interaction, $F(1,25) = 12.33, p = 0.0017$. Follow-up analyses showed a significant decrease in theta power following the US in the unpaired group. Analysis of the prefrontal US theta power revealed a Period effect, $F(1,24) = 32.36, p < 0.0001$. Both groups showed a decrease in theta power in the US period compared with baseline. Analysis of the amygdalar US
theta power revealed a Period effect, $F(1, 19) = 18.80, p = 0.0004$. Overall, these analyses show that the mPFC and amygdala in both groups exhibited a decrease in theta power in the US period compared with baseline. Hippocampal theta power did not change in the US period for the trace group, but decreased in the unpaired group.

Similar analyses were conducted on the relative theta power in the hippocampus, mPFC, and amygdala during the trace interval. No increases or decreases in theta power were observed in the trace interval for any site in either group. Furthermore, there were no differences between the groups in the amount of overall theta power measured during the baseline period.

4.3.4 Coherence of theta rhythmic activity during the trace interval

Coherence analyses were performed on field activity in the hippocampus, mPFC, and amygdala to determine whether these sites exhibit coordinated theta activity during trace fear conditioning. Coherence measures the degree to which two oscillations maintain a constant phase relationship at a specific frequency. Figure 4-6 shows theta coherence between the hippocampus and prefrontal EEG signals. Part A of this figure shows the mean percent change in theta coherence in each 5 s bin of trace or unpaired conditioning on day 3. The period following the US is not shown. Trace conditioned animals showed an increase in coherence during the CS and trace interval, whereas unpaired conditioned animals showed an increase in coherence during the CS, but not in the period following the CS. Part B of Figure 4-6 shows the mean percent change in coherence during the trace interval on each day of training. This figure shows that the
trace group exhibited greater hippocampal-prefrontal coordination during the trace
interval compared with the unpaired group across days of training. A repeated-measures
ANOVA (Training Group X Day) was conducted on the first three days in Figure 4-6B.
This analysis revealed an effect of Training Group, $F(1,24) = 4.85, p = 0.0375$. No
significant Training Group differences were found during the CS or post-US (5-10s)
periods (US data not shown). Further analyses examined the relationship between the
percent change in trace-interval coherence averaged across 4 days and the change in HR
during the trace interval. A significant correlation was found between the coherence and
HR measures in the trace group, $r = 0.65, p = 0.0063$. This suggests that coordination of
hippocampal and prefrontal theta activity during the trace interval is necessary for the
CS-US association to occur. No significant correlation was found in the unpaired group,
$r = -0.14, p = 0.7112$. 
Figure 4-6: *Increased hippocampus-mPFC coherence during the trace interval.*

A. Each point represents the mean percent change in theta coherence between the hippocampus and prelimbic in a 5-s bin averaged across 8 trials on day 3 of trace fear conditioning or unpaired control training. Both groups showed an increase in theta coherence during the CS, but only the trace group showed an increase in coherence during the trace interval. B. Each point represents the mean percent change in theta coherence in the middle 10 s of the trace interval, averaged across 8 trials on each day of trace fear conditioning or unpaired control training. This graph shows that trace conditioned animals exhibited an increase in theta coherence between the hippocampus and mPFC during the trace interval. Error bars = S.E.M.

Coherence analyses during the trace interval revealed that the trace group also showed coordinated theta activity between the hippocampus and amygdala late in training. Figure 4-7 shows the theta coherence between the hippocampus and amygdala. Part A of this figure shows the mean percent change in coherence in 5-s bins on the retention day following trace or unpaired conditioning. This figure shows that theta coherence during the trace interval increased in the trace group. Part B of Figure 4-7 shows the mean percent change in theta coherence during the trace interval across days of training. This figure shows that theta coherence was similar in trace and unpaired animals early in training. Later in training, trace conditioned animals showed increasing trace interval coherence, while unpaired animals remained near baseline levels. A
repeated-measures ANOVA (Training Group X Day) was conducted on the data in Figure 4-7B. This analysis revealed an effect of Training Group that approached significance $F(1,19) = 3.92$, $p = 0.0624$. No significant Training Group differences were found during the CS or post-US (5-10s) periods (US data not shown). Further analyses examined the relationship between the trace interval coherence and changes in HR. The correlation approached significance in the trace group, $r = 0.52$, $p = 0.0664$. No significant correlation was found in the unpaired group, $r = 0.26$, $p = 0.5406$.

Figure 4-7: *Increased hippocampus-amygdala coherence during the trace interval.*

A. Each point represents the mean percent change in theta coherence between the hippocampus and amygdala in a 5-s bin averaged across 8 CS-alone retention trials following trace fear conditioning or unpaired control training. Both groups showed an increase in theta coherence during the CS, but only the trace group showed an increase in coherence during the trace interval. B. Each point represents the mean percent change in theta coherence in the middle 10 s of the trace interval, averaged across 8 trials on each day of trace fear conditioning or unpaired control training. This graph shows that trace conditioned animals showed a trend towards increased theta coherence between the hippocampus and amygdala late in training. Error bars = S.E.M.

Coherence analyses during the trace interval did not reveal a consistent pattern of coordinated theta activity between the mPFC and amygdala across days of trace fear conditioning. Figure 4-8 shows the theta coherence between the mPFC and amygdala. Part A of this figure shows the mean percent change in coherence in 5-s bins on day 1 of
trace or unpaired conditioning. This figure shows that the trace group showed a small increase in theta coherence during the trace interval. Part B of Figure 4-8 shows the mean percent change in theta coherence during the trace interval across days of training. This figure shows that changes in theta coherence in trace and unpaired animals did not show a consistent pattern across days. Analyses on all 4 days in figure 4-8B showed no effect of Training Group or Day. No significant Training Group differences were found during the CS or post-US (5-10s) periods (US data not shown). No significant correlations were found in the trace, $r = 0.127$, $p = 0.7098$, or the unpaired group, $r = -0.31$, $p = 0.4991$.

Figure 4-8: Increased prefrontal-amygdala coherence during the trace interval.

A. Each point represents the mean percent change in theta coherence between the mPFC and amygdala in a 5-s bin averaged across 8 trials on day 1 of trace fear conditioning or unpaired control training. Both groups showed an increase in theta coherence during the CS, and the trace group showed a small increase in coherence during the trace interval. B. Each point represents the mean change in theta coherence in the middle 10 s of the trace interval, averaged across 8 trials on each day of trace fear conditioning or unpaired control training. This graph shows that trace conditioned animals did not exhibit a consistent pattern of theta coherence during the trace interval across days. Error bars = S.E.M.

Coherence analyses focusing on theta activity showed that the hippocampus coordinates with the mPFC early in acquisition and the hippocampus coordinates with the
amygdala late in acquisition and during retention. Figure 4-9 shows the spectral coherence across a trial for one of the rats in the trace conditioning group that showed this pattern of coherence across days. Part A of this figure shows an increase in theta coherence between the hippocampus and prefrontal signals during the trace interval on day 3 for this rat. Part B of this figure shows an increase in the theta coherence between the hippocampus and amygdala signals during and following the CS on the retention day.

![Figure 4-9: Theta coherence for an exemplary trace-conditioned animal.](image)

The color plots show the spectral coherence of two EEG signals across a 57-s trial. Coherence was calculated in sliding 5-s windows (1-s increments) across a single trial for an example rat during trace fear conditioning. Grey boxes denote the presentation of the 5-s CS and filled arrowheads mark the point at which the US was delivered. US artifact was omitted before coherence calculations were performed. No USs were delivered on the retention day, but the open arrowhead marks the latency at which the US had been delivered on previous training days. Coherence values fall in the range 0 (no correlation) to 1 (synchronous).
4.4 Discussion

This study recorded EEG field activity simultaneously in the hippocampus, mPFC, and basolateral amygdala during trace fear conditioning or unpaired control training. EEG activity in the hippocampus and prelimbic area of the mPFC of trace-conditioned animals showed learning-related increases in theta coherence during the trace interval on days 2 and 3 of trace conditioning. Field activity in the hippocampus and amygdala of trace-conditioned rats showed a similar increase in coherence during the trace interval that developed late in training. No consistent learning-related changes in theta coherence were observed between the mPFC and amygdala. These results suggest that the hippocampus and mPFC coordinate activity early during trace fear conditioning to encode the trace interval, while that trace-interval coordination between the hippocampus and amygdala may be important for stable expression and retention of conditioned responses.

The coordination of field activity in the hippocampus and mPFC during the trace interval builds upon the results of our previous single neuron recording experiments in Chapters 2 and 3. These studies showed that single neuron activity in both the hippocampus and mPFC exhibit learning-related changes in activity to the CS and US during trace fear conditioning. Chapter 3 also showed that a subset of prelimbic single neurons exhibit tonic increases in activity following the CS that may serve to bridge the trace interval. This chapter provides further support for the notion that the hippocampus and mPFC encode the different elements of the CS-trace-US association, suggesting that these two structures communicate during the trace interval. Several recent studies have
demonstrated a similar hippocampal-prefrontal coordination during spatial working memory tasks (Hyman, Zilli, Paley, & Hasselmo, 2005; Jones & Wilson, 2005a, 2005b; Siapas, Lubenov, & Wilson, 2005). The Jones and Wilson (2005b) study showed that hippocampal-prefrontal coordination was stronger on trials where the animal made correct responses than on error trials. This parallels our findings that showed a significant correlation between hippocampal-prefrontal coherence and conditioned HR in trace-conditioned animals. This suggests that hippocampal-prefrontal coordination during the trace interval is important for forming CS-US associations and expressing learned fear responses.

During the later stages of training, the hippocampus and amygdala showed increased theta coherence during the trace interval. This finding suggests that coordination of activity between these two structures is important after conditioned responding is established. The amygdala is the major output structure in fear conditioning networks, with projections to multiple downstream sites important for the production of conditioned and unconditioned fear responses (LeDoux, Iwata, Cicchetti, & Reis, 1988). It is possible that the hippocampus interacts with the amygdala to modify or strengthen the conditioned response after the CS-trace-US association is established. Along the same lines, two recent studies by Pape and colleagues suggest that coordination between the hippocampus and amygdala may be important for the consolidation or retention of fear memories (Pape, Narayanan, Smid, Stork, & Seidenbecher, 2005; Seidenbecher, Laxmi, Stork, & Pape, 2003). These studies examined rhythmic activity in the hippocampus and amygdala during a differential delay fear conditioning task in mice and showed that field activity in these sites is coordinated
on CS+ presentations several hours after fear conditioning, but not immediately after fear conditioning (Pape, Narayanan, Smid, Stork, & Seidenbecher, 2005; Seidenbecher, Laxmi, Stork, & Pape, 2003). Coordination between the hippocampus and amygdala in this study occurred specifically during the trace interval. This may suggest that the trace interval is a significant component of the trial when refining conditioned responses late in training (e.g., appropriately timed responses) or when consolidating trace fear memories.

Theta coherence of field activity in the hippocampus, mPFC, and amygdala increased during the CS. This increase was observed in both the paired and unpaired groups on all days of training. These findings suggest that increased theta coherence during the CS reflects a more general coordination of activity within the fear network in response to salient stimuli. In the unpaired pseudoconditioning procedure, the CS and US are not paired, but they occur in the same context. Therefore, the CS is not completely neutral and it is possible that increased theta coherence during the CS reflects an orienting response or increased awareness in both groups.

Early in training, the hippocampus and amygdala of trace-conditioned animals showed moderate increases in theta power during the CS. These increases occurred on day 1 for the amygdala and on day 2 for the hippocampus. It is possible that these early theta power increases reflect the initial stimulus encoding by the hippocampus and amygdala. Our previous hippocampal single neuron recording study in Chapter 2 provides some support for this. Chapter 2 showed that single neurons in the CA1 region exhibited an increase in activity early in trace fear conditioning, but exhibited decreases in activity later in training. Hippocampal and amygdalar responses to stimuli occur early in the learning process during trace fear conditioning in humans (Buchel, Dolan,
Aronomy, & Friston, 1999; Knight, Cheng, Smith, Stein, & Helmstetter, 2004). The increases in theta power in this chapter are consistent with the notion that the hippocampus and amygdala participate in early encoding of the CS during trace fear conditioning.
Chapter 5

General Discussion

This dissertation showed that neuronal activity in the hippocampus coordinates with the activity in the medial prefrontal cortex to encode information during trace fear conditioning. Chapter 2 showed that single neurons in the dentate gyrus (DG) and CA1 areas of the hippocampal formation encode learning-related information about the CS and US, but not the trace interval. Chapter 3 showed that single neurons in the prelimbic area of the medial prefrontal cortex (mPFC) exhibit sustained increases in activity that may bridge the trace interval. Chapter 4 showed that rhythmic field activity in the hippocampus and the mPFC is coordinated at theta frequencies during the trace interval. Together, the experiments in this dissertation provide support for the cooperative encoding of the CS-US association by the hippocampus and mPFC across a 20-s trace interval.

Prior to the start of this dissertation work, very little was known about the neuronal basis of trace fear conditioning. In recent years, trace fear conditioning has become more widely used in studies examining various aspects of hippocampal function and hippocampus-dependent learning. As a result, there is considerable interest in understanding the neural processes underlying trace fear conditioning. The studies in this dissertation provide the first steps towards characterizing the single-neuron and population activity that governs trace fear conditioning, and suggest that activity in
multiple sites within a learning network contributes to the acquisition of trace fear conditioning.

This dissertation provides a general network model of neuronal activity underlying trace fear conditioning. This model proposes that during the conditioning process, the hippocampus encodes learning-related information about the CS and US and communicates stimulus information to the mPFC. The mPFC in turn maintains a CS-representation across the trace interval so that a Hebbian-like overlap of the CS and US can occur. This aspect of the model is supported by Chapters 2 and 3. Chapter 2 showed that hippocampal single neurons encode learning-related information about the CS and US early in training, and Chapter 3 showed that single neurons in the prelimbic area of the mPFC exhibit sustained increases in firing during the 20-s trace interval. The DG and CA1 regions of the hippocampus may assess the significance of the stimuli and send CS-related information to the mPFC to initiate the bridging process. Chapter 4 adds to this network model and suggests that coordination of population field activity in the hippocampus and mPFC may be important for initiating or maintaining the bridging activity. Chapter 4 also shows that hippocampal-prefrontal coordination during the trace interval occurs on a time-scale similar to the stimulus and trace interval encoding found in Chapters 2 and 3, i.e. early in training. Furthermore, coordination of activity in the hippocampus and amygdala may occur later in training to refine the conditioned responses as the CS-trace-US association is strengthened.

Previous models of trace conditioning hypothesized that activity in the hippocampus bridges the trace interval (e.g., Levy, Sanyal, Rodriguez, Sullivan, & Wu, 2005; Rodriguez & Levy, 2001; Wallenstein, Eichenbaum, & Hasselmo, 1998). These
models were based on trace eyeblink paradigms with trace intervals < 1 s. Chapter 2 provides evidence that the DG and CA1 regions of the hippocampus do not bridge the trace interval. Instead, the studies in this dissertation suggest that the hippocampus and mPFC work together to bridge the trace interval. This network model is consistent with the results of hippocampal and prefrontal lesions in trace conditioning tasks, which show that lesions of either structure impair the acquisition of trace conditioning (e.g., McEchron, Bouwmeester, Tseng, Weiss, & Disterhoft, 1998; McLaughlin, Skaggs, Churchwell, & Powell, 2002; Moyer, Deyo, & Disterhoft, 1990; Quinn, Oommen, Morrison, & Fanselow, 2002; Solomon, Vander Schaaf, Thompson, & Weisz, 1986; Tseng, Guan, Disterhoft, & Weiss, 2004). Cooperative encoding of the trace interval by the hippocampus and mPFC is supported by recent studies that showed coordination between these structures during spatial working memory. Spatial working memory tasks require an animal to use spatial information acquired before a delay period to guide behavior after the delay. As in trace conditioning, lesions of either the hippocampus or the mPFC impair performance in these tasks (Floresco, Seamans, & Phillips, 1997; Granon, Vidal, Thinus-Blanc, Changeux, & Poucet, 1994; Seamans, Floresco, & Phillips, 1995). Recent recording studies have shown that prefrontal activity becomes synchronized with hippocampal theta activity during phases of spatial learning that require working memory (Jones & Wilson, 2005a, 2005b; Siapas, Lubenov, & Wilson, 2005). These findings support a network model where the hippocampus coordinates with the mPFC to maintain hippocampus-encoded information (e.g. auditory-CS or spatial cues) over a temporal interval.
The network model supported by the results of this dissertation suggests that the hippocampus coordinates with the mPFC to maintain a representation of the CS across the trace interval. It is unclear from these studies how CS-related information is transferred back to the hippocampus or to the amygdala in order to overlap with the US. The mPFC does not send direct projections back to the hippocampus, but prefrontal activity could impact hippocampal activity indirectly, along thalamic or amygdalar pathways (Vertes, 2004, 2006). The mPFC does send direct excitatory projections to the basolateral amygdala (Likhtik, Pelletier, Paz, & Pare, 2005; McDonald, Mascagni, & Guo, 1996; Vertes, 2004, 2006). Activity in the prefrontal-amygdala pathway has been shown to modulate neuronal responses to conditioned stimuli (Rosenkranz, Moore, & Grace, 2003). We hypothesized that the mPFC and amygdala would exhibit coordinated activity during the trace interval that would provide a mechanism for CS-US overlap in the trace fear paradigm. The results of Chapter 4, however, showed that the mPFC and amygdala do not exhibit consistent theta coherence across days. This finding may suggest that large-scale coordination of populations of neurons is not required for the transfer of CS-related information in the trace fear network. Whereas hippocampal-prefrontal coordination may be required throughout the trace interval, it is possible that prefrontal-amygdala communication is necessary only near the end of the trace interval. In this case, individual mPFC neurons could activate a subset of amygdala neurons near the time of US delivery. Future studies should examine single neuron activity in the amygdala to determine whether amygdala neurons exhibit increased firing in anticipation of the US.
Coordination of the hippocampus and amygdala in this network model of trace fear conditioning may be important for the expression or modulation of conditioned HR responses after the CS-trace-US association is established. The amygdala mediates the expression of conditioned fear responses through projections to the hypothalamus and brainstem structures necessary for generating autonomic and defensive behaviors (LeDoux, Iwata, Cicchetti, & Reis, 1988). As a result, interactions between the amygdala and other learning structures have the capacity to modulate fear responses. During the later stages of trace fear conditioning when the CS-trace-US association is strongest, coordinated hippocampal-amygdalar activity may modify the fear responses to be more specific to the trace interval. In support of this, Chapter 4 showed a correlation between hippocampal-amygdalar theta coherence and conditioned tachycardia during the trace interval.

The network model proposed in this dissertation is only a general framework and does not describe other aspects of the conditioning process, such as extinction. A number of studies have shown that the mPFC is a key site of fear extinction learning, and activity in the mPFC-amygdala pathway modulates fear expression during the retention and extinction of fear memories (Corcoran & Quirk, 2007; Milad & Quirk, 2002; Milad, Vidal-Gonzalez, & Quirk, 2004; Quirk, Garcia, & Gonzalez-Lima, 2006; Rosenkranz, Moore, & Grace, 2003; Vidal-Gonzalez, Vidal-Gonzalez, Rauch, & Quirk, 2006). It is possible that in the trace fear paradigm, coordination of prefrontal and amygdalar activity may be more important during extinction processes rather than during acquisition. Recent studies have also shown that inactivation of the hippocampus impairs the retention of extinction learning (Corcoran, Desmond, Frey, & Maren, 2005; Corcoran &
Maren, 2004), and both the hippocampus and mPFC are activated during recall of fear extinction in humans (Kalisch et al., 2006; Milad et al., 2007). Based on these findings, it is likely that the three major structures in the trace fear network exhibit patterns of coordinated activity during extinction as well as during trace fear acquisition. Future studies that examine activity in the trace fear network should include several days of extinction training to determine how these structures interact during the acquisition, retention, and extinction phases of trace fear conditioning. Although other aspects of the model need to be elucidated, this model nonetheless describes a mechanism by which the hippocampus and mPFC cooperatively encode information about trace fear conditioning.
Bibliography


Collins, D. R. & Pare, D. (2000). Differential fear conditioning induces reciprocal changes in the sensory responses of lateral amygdala neurons to the CS(+) and CS(-). *Learning and Memory, 7*(2), 97-103.


McEchron, M. D., Alexander, D. N. & Gilmartin, M. R. (*in prep*). The time-course and dynamics of trace classical conditioning of heart rate responses in restrained and freely moving rats.


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