ARC PROTEIN ENHANCEMENT IN THE DORSAL AND VENTRAL HIPPOCAMPUS FOLLOWING TRACE, CONTEXTUAL, AND DELAY FEAR CONDITIONING IN A NOVEL OR FAMILIAR CONTEXT

By

CALEB DENEB HUDGINS

A dissertation submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

Graduate Program in Psychology

Written under the direction of

Timothy Otto, PhD

And approved by

_____

_____

_____

_____

New Brunswick, New Jersey

January 2017

ABSTRACT OF DISSERTATION

Arc protein enhancement in the dorsal and ventral hippocampus following trace, contextual and delay fear conditioning in a novel or familiar context

By CALEB DENEB HUDGINS

Dissertation Director:

Professor Timothy A. Otto

Arc (Activity-regulated cytoskeleton-associated protein) is an effector neuronal immediate-early gene (IEG) and has been closely linked to behaviorally-induced neuronal plasticity. The present experiments are the first to characterize the regional distribution of Arc expression induced by hippocampal-dependent learning. More specifically, these studies examined the regionally-selective, dissociable patterns of Arc expression induced by Pavlovian trace fear conditioning, delay fear conditioning, and contextual fear conditioning as well as novel context exposure.

This research was guided by anatomical studies identifying heterogeneity of connectivity across the transverse (CA1, CA3) and septo-temporal (dorsal vs. ventral) axes of the hippocampus; companion neuropsychological experiments suggest that these subregions likely play functionally dissociable roles in different forms of hippocampal-dependent learning. Hence the primary goal of the present study was to characterize the expression of Arc protein across both the

septotemporal and transverse axes of the hippocampus induced by hippocampal dependent trace fear conditioning and compare these expression patterns to those induced by other fear conditioning paradigms. A second goal of these studies was to explore which specific paradigmatic features of the trace fear conditioning task itself are responsible for the observed patterns of Arc expression. These goals were accomplished by directly comparing behavior and Arc protein expression patterns following trace fear conditioning to that following novel context exposure, contextual fear conditioning, and delay fear conditioning in either a novel or familiar context.

The results of these studies suggest that, within the dorsal hippocampus, Arc expression in CA3 induced by trace fear conditioning may play a unique role in representing the context, while Arc protein expression within ventral CA3 may reflect CS processing. Arc protein expression in dorsal and ventral CA1 are likely not meaningfully involved in trace fear conditioning as there is either a lack of significant enhancement (dorsal CA1) or enhancement is not unique to subjects trained in trace fear conditioning (ventral CA1). The specific regional pattern of Arc protein enhancement induced by trace fear conditioning may reflect the unique temporal parameters of the task which critically engages the hippocampus in processing both contextual representations as well as the explicit CS. This additional hippocampal processing may account for the greater enhancement in Arc protein in dorsal and ventral CA3 for subjects trained in trace fear conditioning compared to novel context exposure, or contextual and delay fear conditioning.

TABLE OF CONTENTS

Chapter 1: GENERAL INTRODUCTION

The investigation of the neurobiological basic of learning and memory has been fundamentally shaped by identifying the relationship between particular brain structures and pathways and how they influence certain kinds of learning. One of the more commonly studied structures in this monumental endeavor is a subcortical structure within the medial temporal lobe of the mammalian brain: the hippocampus. Some of the first and most highly cited evidence that the hippocampus is involved in memory was described in the case of HM (Scoville & Milner, 1957; Milner et al., 1968), whose hippocampus and much of the medial temporal lobe was removed as a treatment for protracted epileptic seizures. Initial neuropsychological examination suggested that HM's surgery resulted in profound and global anterograde amnesia (Scoville & Milner, 1957); subsequent study however revealed that HM retained the capacity for certain kinds of memory (Milner et al., 1968; Cohen & Squire, 1980). These observations gave rise to the notion that different kinds or "types" of memory are likely neuroanatomically dissociable.

Based in part on these observations, thousands of studies over the last 50 years examined how different kinds of learning are impaired by interfering with hippocampal activity (Squire et al., 2004; Eichenbaum, 1999; Andersen et al., 2006). This research was complemented by anatomical studies identifying heterogeneity of connectivity across the transverse (DG, CA1, CA2, CA3) and septo-temporal (dorsal vs. ventral) axes of the hippocampus (Amaral & Lavenex, 2007; Risold & Swanson, 1996). Emerging evidence from these studies strongly

suggests that the hippocampus is functionally dissociable along both axes with activity within different regions supporting different kinds of learning (Moser and Moser, 1998; Guzowski et al., 2004; Eichenbaum et al., 1996; Czerniawski et al., 2009; Hunsaker & Kesner, 2008). These distinctions, reviewed below, are based in part on retrograde and anterograde tracing studies examining hippocampal/cortical connectivity representing unique aspects of the sensory world (Risold & Swanson, 1996), as well as hippocampal/subcortical connectivity influencing behavioral output (Pitkanen et al., 2000; Risold & Swanson, 1996).

Anatomical and functional dissociations along the septo-temporal axis of the hippocampus

Mounting evidence suggests that the dorsal and ventral hippocampus are both functionally and anatomically dissociable. Specifically, the dorsal hippocampus receives its input from the entorhinal cortex which in turn receives prominent projections from primary sensory cortical areas (reviewed in Moser & Moser, 1998; Pitkanen et al., 2000); this pattern of connectively is reflected in this regions' involvement in spatial learning tasks. An intriguing reflection of the consolidation of multimodal sensory information is the development of "place fields" which are sets of cells within the hippocampus that are only active when an animal is occupying a particular location in space. These location sensitive "place cells" form reliably from a variety of combinations of sensory input (Zhang & Manahan-Vaughan, 2015) and are implicated in spatial and contextual learning tasks (O'Keefe & Dostrovsky, 1971; O'Keefe & Nadel, 1978; Eichenbaum et al., 1996; Knierim et al., 1995). Although "place cells" have also been identified in the

ventral hippocampus, their numbers are limited and their spatial selectivity is dramatically reduced (Jung et al., 1994).

Unlike the dorsal hippocampus, the ventral hippocampus has strong monosynaptic and reciprocal connections with the amygdala, and correspondingly has been implicated in certain aspects of emotional learning (Yoon & Otto, 2007; Rogers et al., 2006; Rudy & Matus-Amat, 2005; Maren & Holt, 2004). Previous research from our laboratory has provided strong evidence for a functional double dissociation between the dorsal and ventral hippocampus. We have shown that inactivation of the dorsal hippocampus produced explicit memory deficits in a spatial alternation task, but had no effect on the acquisition of trace fear conditioning. Conversely, inactivation of the ventral hippocampus resulted in dramatic impairments in trace fear conditioning with no effect on spatial alternation (Czerniawski et al., 2009). These distinctions are discussed in more detail below with respect to the involvement of the hippocampus in several different fear conditioning paradigms.

Anatomical and functional dissociations along the transverse axis of the hippocampus

Accumulating evidence suggests that hippocampal subfields CA1 and CA3 may also play dissociable roles in learning (Gilbert & Kesner, 2004; Vago et al., 2007; Hoge & Kesner, 2007; Hunsaker & Kesner, 2008; Goodrich et al., 2008; Hunsaker et al., 2006; Lee et al., 2005). In this regard, most of the research investigating functional and anatomical differences between the CA1

and CA3 subfields has focused on the dorsal hippocampus. The results of these studies collectively suggest that hippocampal subfield CA3 forms an extensive "autoassociative network" (Hoang & Kesner, 2008; Rolls & Kesner, 2006; McNaughton & Morris, 1987) in which CA3 maintains recurrent collateral connections as well as prominent projections to CA1 along the dorsal-ventral span of the hippocampus (Amaral & Lavenex, 2007). Lesions of this region impair performance on "pattern completion" tasks (reviewed in Kesner, 2007). The CA1 subregion, which receives input primarily from the entorhinal cortex and from other hippocampal regions, including CA3 (reviewed in Moser & Moser, 1998; Pitkanen et al., 2000), has been implicated in tasks requiring "temporal processing," (Hoge & Kesner, 2007), while both CA1 and CA3 seem to be necessary for contextual fear conditioning (Hunsaker & Kesner, 2008), temporal sequence of spatial locations (Hunsaker et al., 2008), and temporal pattern completion (Hoang & Kesner, 2008).

While functional dissociations among the transverse subfields within ventral hippocampus have received considerably less experimental attention, it is clear that ventral CA1 establishes dense and reciprocal connections to the central and accessory basal amygdala regions (Canteras & Swanson 1992; Pitkanen et al., 2000) while CA3 receives mostly afferent input from basal amygdala (Pitkanen et al., 2000). Consistent with these anatomical projections, recent evidence suggests that lesions of ventral CA3 produce impairments in tasks which involve input from the amygdala to the hippocampus (Hunsaker & Kesner, 2008).

While a great number and variety of behavioral tasks have been used to investigate hippocampal function, some of the strongest evidence of functional dissociations within hippocampal neurocircuitry can be found within variations of Pavlovian fear conditioning paradigms, specifically auditory trace fear conditioning, delay fear conditioning, and contextual fear conditioning

Pavlovian trace and delay fear conditioning

Pavlovian conditioning involves the pairing of a neutral conditioned stimulus (CS), with an unconditioned stimulus (US), which elicits an unconditioned response (UR). After pairing, presentation of the CS alone in a novel environment typically elicits a conditioned response (CR) similar in topography to the UR. Generally, the level of conditioned responding elicited by the CS is thought to reflect the strength of CS-US association acquired during conditioning. Research on Pavlovian fear conditioning utilizes an aversive US, most commonly a mild footshock, which elicits a variety of conditioned responses (LeDoux, 2003). These responses are measured in a variety of different ways (see Kim & Jung, 2006 for review), but the most frequently measured CR is “freezing” behavior (Izquierdo et al., 2016; Fanselow 1980).

The differences between trace fear conditioning (TFC) and delay fear conditioning (DFC) are defined by the temporal parameters of CS and US presentation, as well as the “amount “ of conditioned responding to the CS during subsequent testing. More specifically, during TFC there is a temporal gap (trace interval) between CS offset and US onset, while during DFC the CS overlaps

with and co-terminates with the US. During subsequent testing to the explicit CS, animals trained in TFC typically exhibit lower levels of the freezing CR relative to animals previously trained in DFC (Bolles et al., 1978); Interestingly, while freezing elicited by the explicit CS during testing following TFC can often be quite low, data from our laboratory has shown that freezing during the trace interval is considerably more robust, typically as high as that elicited by the CS following DFC (Czerniawski et al., 2009; 2011; 2012; Chia & Otto, 2013). Many theories have been proposed to account for this difference in CS learning, including the initial notion of the memory "trace" of the CS, not the CS itself, is paired with the US and in turn produces a weaker memory (Pavlov, 1927), weaker representation of the CS-US association (Rescorla and Wagner, 1972), or the more recent appeals to differential learning of temporal intervals between events (Matzel, Held, & Miller, 1988; Cole et al 1995, Gallistel & Gibbon, 2000).

Data from our lab as well as others have demonstrated that the acquisition of TFC is compromised following ventral hippocampal inactivation via excitotoxic lesions (Yoon & Otto, 2007), inactivation with regionally selective infusion of the GABA-A agonist muscimol (Czerniawski et al., 2009), infusion of anti-sense oligodeoxynucleotides (ODNs) to the immediate early gene Arc (Czerniawski et al., 2011), or NMDA receptor antagonists (APV) (Czerniawski et al., 2012). Interestingly, the acquisition of TFC is generally unaffected by lesions or inactivation of dorsal hippocampus (Otto & Yoon, 2007; Czerniawski et al., 2009). By contrast, acquisition of TFC is dramatically impaired by manipulations of dorsal hippocampus that are known to affect neuronal plasticity, for example

infusion of APV or Arc ODNs, without impairing normal cell activity during conditioning (Czerniawski et al., 2011; Czerniawski et al., 2012). These data suggest that while the dorsal hippocampus is may not be necessary for learning TFC, if these neurons are functioning normally during conditioning but neuronal plasticity is blocked, a learning deficit is observed.

While the acquisition and/or retention of TFC can be impacted by a variety of hippocampal manipulations, performance in DFC is largely unaffected following either dorsal hippocampus inactivation (Corcoran & Maren, 2001; Misane et al., 2005; Hunsaker & Kesner, 2008; Parsons & Otto, 2008) or by impairing neuronal plasticity in the ventral hippocampus (Czerniawski et al., 2011). The general paradigmatic similarity between DFC and TFC, yet the unique recruitment of the hippocampus, and particularly the ventral hippocampus, for TFC, makes these tasks ideal for comparing hippocampal involvement in fear conditioning by identifying regions and subfields within the hippocampus undergoing learning related plasticity. These tasks have the added benefit of being rigorously investigated by experimental psychologist for decades, allowing a better understanding of the ways in which the parameters of conditioning tasks can be manipulated to affect learning.

Pavlovian contextual fear conditioning

Pavlovian conditioning is either cued, with an explicit CS such as a tone or light as in TFC and DFC, or uncued, in which only the conditioning context itself is paired with the US (context-US association). Contextual fear conditioning

(CFC), as well as other forms of contextual learning, is in most cases hippocampal-dependent, and as such is similar to trace fear conditioning (Phillips & LeDoux 1992; Maren et al., 1997; Kim & Fanselow 1992; Parsons & Otto 2009; Frankland et al., 1998). As discussed above, during context exploration, place fields develop within different populations of neurons within hippocampal subfields, and are thought to reflect the animal's location in space. Hippocampal activity of this sort has been implicated in CFC as this activity can be inhibited to induce a learning deficit (Tanaka et al., 2014), yet can also be optogenetically marked and reactivated to produce an artificial contextual memory (Ramirez et al., 2013). Importantly, contextual fear learning occurs during both DFC and TFC as well. That is, following training in either DFC or TFC, animals returned to the training context will exhibit a CR in the absence of the explicit CS, suggesting that during both DFC and TFC, animals acquire both CS-US and Context-US associations.

These same tasks which are used to investigate the functional dissociations within the hippocampus have also been used to explore the molecular mechanisms supporting hippocampal-dependent learning (Bauer et al. 2002; Lonergan et al., 2010; Ramamoorthi et al., 2011; Czerniawski et al., 2012). One such proposed mechanism is the selective strengthening of the synapses activated during, and as a result of learning, which is well modeled by the laboratory phenomenon typically referred to as "long-term potentiation" (Lomo 1966; Bliss & Lomo 1973).

Synaptic plasticity and learning

Synaptic plasticity, and specifically long term potentiation (LTP), is proposed physiological mechanism to account for changes in neuronal strength induced by neuronal activity, and has emerged as a prominent model for the neural processes potentially subserving some forms of learning (Bliss & Collingridge 1993). Often referred to as Hebbian plasticity, this learning related change in synaptic strength provides a theoretical basis of how neuronal ensembles are formed and are able to change their activity in order to represent the changing outside world (Hebb, 1949). Within the hippocampus, synaptic plasticity is driven, in part, by regulation of AMPA receptor number and sensitivity which is modulated by a cascade of intracellular reactions following strong bouts of afferent activation (see Herring & Nicoll, 2016 for a review). While a whole host of molecular mechanisms are involved in different forms of synaptic plasticity (Korb & Finkbeiner 2011), certain proteins seem to be uniquely involved in learning and memory (Shepherd & Bear, 2011). One such protein is activity-related cytoskeletal protein (Arc).

Arc protein and synaptic plasticity

Arc is an effector neuronal immediate-early gene (IEG) (Shepherd & Bear, 2011). Neuronal IEGs encode transcription factors, cytoskeletal proteins, growth factors, metabolic enzymes, and other proteins involved in signal transduction and other downstream cascades of gene expression (Lanahan & Worley, 1998). Because Arc has low basal levels of expression, it is easy to detect changes in Arc protein levels following a variety of neuronal or behavioral manipulations (Vazdarjanova & Guzowski, 2004). Accumulating evidence suggests that the

expression of Arc is tightly coupled with the induction and expression of late phase LTP (L-LTP) and the formation of many forms of hippocampal-dependent memory. For example, L-LTP, Arc transcription, and hippocampal dependent memory are all typically NMDA receptor dependent and are induced by similar forms of neuronal activity (Guzowski, 2002; Czerniawski et al., 2012; Chia & Otto, 2013). Within the amygdala (LeDoux, 1995) and hippocampus, two brain regions implicated in Pavlovian fear conditioning, LTP is mediated, in part, by NMDA receptor-mediated calcium influx (Bliss et al., 2007), and inhibiting Arc translation via anti-sense oligodeoxynucleotides (ODNs) or NMDA receptor antagonists (APV) blocks both L-LTP (Guzowski et al., 2000) and some forms of learning thought to be mediated via naturally-occurring alterations of synaptic strength within the hippocampus (Czerniawski et al., 2012; Chia & Otto, 2013).

Arc protein is also of particular interest due to its unique translation/transcription dynamics. More specifically, Arc mRNA is rapidly and robustly transported to activated dendritic synaptic zones where the protein product associates with pre-existing cytoskeletal proteins, supporting changes in post synaptic cytoskeletal structure and cellular modifications related to long term potentiation (AMPA receptor exocytosis) (Link et al., 1995; Lyford et al., 1995; Steward et al., 1998). Arc expression has also been more closely linked to neuronal activity which induces synaptic plasticity as opposed to neuronal firing *per se* (Fletcher et al., 2006). Finally, Arc protein is also implicated in homeostatic plasticity (Verde et al., 2006; Shepherd et al., 2006) as well as long term depression (LTD) (Waung et al., 2008) via AMPA receptor endocytosis.

Synaptic plasticity and arc protein expression in the hippocampus

Several previous studies have explored the extent to which the expression of Arc protein in the hippocampus can be modified by experience (Ramirez-Amaya et al., 2005; Monti et al., 2010; Li et al., 2009). While these studies have identified a number of temporal and experiential factors contributing to hippocampal Arc expression, until recently none have examined the relationship between Arc expression and forms of learning known to depend critically on hippocampal integrity. However, our laboratory has recently found that hippocampal-dependent TFC dramatically enhances both Arc mRNA and protein within dorsal and ventral hippocampus, and that both TFC and the associated learning-related enhancement of Arc can be blocked by infusing either Arc antisense ODNs or the NMDA receptor antagonist APV into the dorsal or ventral hippocampus prior to training (Czerniawski et al., 2011; Czerniawski et al., 2012).

Evidence from immunohistochemical studies suggests that Arc is differentially expressed across the transverse axis of the hippocampus following a variety of manipulations thought to require hippocampal processing (Beer et al., 2013; 2014). These studies suggest that Arc protein expression in dorsal CA1 and not CA3 are considered to reflect both spatial and non-spatial changes in the environment where CA3 is predominately spatially tuned (Beer et al., 2013). While immunohistochemical evidence suggests that novel context exposure (Rameriz-Amaya et al., 2005), exposure to drug cues (Monti et al., 2010; Li et al., 2009), and object exploration (Beer et al., 2013; 2014) enhance Arc protein expression in subfields within the dorsal hippocampus, the extent to which these

exposure paradigms specifically engage the hippocampus in a meaningfully critical manner is unknown.

While these studies clearly suggest that Arc expression within the hippocampus may play a critically important role in the acquisition and retention of some forms of hippocampal-dependent memory, the extent to which Arc is induced differentially across the transverse axis within both dorsal and ventral hippocampus following hippocampal-dependent learning has not been explored. When considered together with the emerging evidence suggesting that the hippocampal subfields along both the septotemporal and transverse axes of the hippocampus likely play dissociable roles in memory (Kesner et al., 2010; Fanselow & Dong, 2010; Hunsaker & Kesner, 2008; Yoon & Otto, 2007; Rogers et al., 2006; Moser & Moser, 1998), an important question remains to be explored: to what extent does the regional expression of Arc reflect the putative roles of each of these subregions in learning? Immunohistochemical procedures allow for the examination of the expression patterns of Arc protein across the various subfields of the hippocampus, thereby permitting a more precise identification of the distribution of neurons activated and potentially undergoing plasticity during a learning experience. Given the evidence from our laboratory and others suggesting functional and anatomical dissociations within the anatomically dissociable subfields of the hippocampus, identifying the specific subfields within which Arc is preferentially expressed following training in a number of tasks known to differentially recruit and require hippocampal activity

will provide evidence addressing the potential role of Arc-dependent neuronal plasticity in these regions for uncompromised task performance.

The present experiments are the first to attempt to characterize the regional distribution of Arc protein expression induced by hippocampal-dependent learning. More specifically, these studies examined the regionally-selective, dissociable patterns of Arc expression induced by Pavlovian trace fear conditioning, delay fear conditioning, and contextual fear conditioning. Hence the primary goal of the present study is to better characterize the expression of Arc protein across both the septotemporal and transverse axes of the hippocampus induced by the acquisition of a form of learning known to be hippocampal dependent (TFC) relative to one known to be hippocampal independent (DFC), and provide more information on specific paradigmatic features of the trace fear conditioning task itself which lead to Arc expression.

Chapter 2: GENERAL METHODS

All procedures have been approved by Rutgers University's Institutional Animal Care and Use Committee (Protocol #96-033).

General Methods

The following methods were used across the following experiments. Experiment-specific variations in methodology are described in detail within the methods section of each experiment.

Subjects. Sprague-Dawley rats (Harlan, Indianapolis, IN), weighing 250-300g, served as subjects. Animals were housed in clear plastic tub cages in an approved animal vivarium. Animals were under a 12 h light/dark cycle with all behavioral procedures occurring during the light cycle. Subjects had access to food and water *ad libitum*.

Apparatus

Behavioral conditioning and testing chambers. Conditioning was conducted in 3 identical behavioral chambers (30 cm x 24 cm x 27 cm), each enclosed in a sound attenuating enclosure (56 cm x 41 cm x 42 cm). The floor of the chambers were composed of 16 stainless steel rods equally spaced by 1.9 cm which were connected to a shock generator (model H13-15, Coulbourn Instruments, Allentown, PA) designed to administer footshock unconditioned stimulus (US) (0.6 mA). Two of the opposing walls were composed of transparent Plexiglas and the other two of aluminum. When appropriate, a computer-generated tone (3.9

kHz, 80 dB) was presented through a speaker mounted 14 cm above the floor on the outside one of the aluminum chamber walls. A single light bulb (29 V, 0.04 A) was located 24.5 cm above the floor and provided continuous illumination. A one-way glass window on the front door of the sound attenuating enclosure allowed an experimenter to observe and score the behavioral measure of freezing using a hand switch that was connected to the computer controlling all paradigmatic events. A motion sensor (Coulbourn Instruments, Allentown, PA) was secured to the top of the chamber to monitor mobility independent of freezing behavior. The training chamber was cleaned with a commercially available cage cleaner (Research Laboratories Inc.) between sessions. The testing session for fear conditioning took place in a novel chamber located in a different experimental room. The testing chamber had the same measurements and configuration as the training chambers but was differentiated from the training chamber in that the entire floor was covered with black Plexiglas and a black and white striped panel was attached to two of the opposing walls. The testing chamber was cleaned with alcohol between sessions.

Procedure

Behavioral procedures

Auditory trace fear conditioning (TFC) was conducted using procedures identical to those used in previous experiments in our laboratory (Czerniawski et al., 2012), and consisted of a 2 minute acclimation period followed by seven pairings of a tone (16 sec, 3.9 kHz, 80 dB) and footshock (2 sec, 0.6 mA), with a trace

interval of 28 seconds between the offset of the tone and onset of the shock and a 2 min ITI (intertrial interval) for a total session length of 19min 22sec.

Contextual fear conditioning (CFC) consisted of a 2 minute acclimation period followed by seven presentations of footshock (2 sec, 0.6 mA) each separated by a 2min 28sec ITI for a total session length of 19min 22sec. CFC task parameters are identical to TFC expect no tone is presented.

Auditory delay fear conditioning (DFC) consisted of a 2 minute acclimation period followed by seven pairings of a tone (16 sec, 3.9 kHz, 80 dB), the last 2 sec of which overlapped and co-terminated with the delivery of footshock (0.6 mA), each separated by a 2min ITI for a total session length of 16min 8sec. DFC task parameters are identical to TFC with the exception of the trace interval, resulting in a shorter total session length.

Across all conditioning tasks, the behavioral response of freezing, defined as a rigid posture and lack of movement except that required for respiration (Fanselow, 1980), was recorded throughout the entire conditioning session by an observer blind to the experimental condition of the subject. These raw data were subsequently transformed into the percentage of time spent freezing during the first ITI (ITI-1), CS (CS-1), and trace interval (TR-1), and the remaining ITIs, CS, and trace intervals of the training session. After conditioning animals were removed and returned to their home cage after which a subset was sacrificed one hour later for tissue processing for immunohistochemical analysis of Arc protein expression.

Home cage: Home cage subjects (HC) were sacrificed directly from their home cage for immunohistochemical after either 5 days (Experiment 1) or 10 days (Experiments 2-4) of handling for 2 minutes per day. Arc expression for these animals was used as the primary control against which Arc protein expression in other groups is compared.

Post-training testing for fear conditioned to the tone and training context: A subset of animals were tested 24 and 48hr after training for CS- and Context-elicited freezing. Specifically, half of the trained animals were first tested 24hr later in a novel context for fear conditioned to the CS followed by a second testing session conducted in the original training context 24hr later (48hr after training) to examine levels of contextually-elicited fear. The testing session examining tone-elicited freezing for animals trained in TFC, DFC, or CFC consisted of one session comprised of three trials. The timing of stimulus delivery and duration of both the CS and ITI were identical to that used during training for the respective conditioning task except that footshock was not presented and the number of trials was decreased to 3 to reduce the potential effect of extinction during testing. As during conditioning, the behavioral measure of freezing was recorded throughout the entire testing session. The first two minutes in the testing chamber was used as a baseline measure of freezing behavior. These raw data were subsequently transformed into the percentage of time spent freezing during the first ITI (ITI-1), and the remaining ITIs, CS, and trace intervals of the testing session. Even though animals trained in CFC were not conditioned

to an auditory CS, this group received this test in order to maintain continuity of testing and environment exposure between the 3 different conditioning tasks.

Conditioned fear to the training context was assessed 24 hours later (48 hours after training) by placing each subject into the chamber in which training occurred for the same 3 trial period as used during CS testing, but no tones were presented. Freezing was recorded continuously during each testing session by an observer blind to the subjects' condition. These raw data were transformed into the percentage of time spent freezing during periods consistent with those during which the ITI, CS, and trace interval are presented during initial training. Freezing behavior between and within training and testing conditions was compared. Data was analyzed using separate one- or two-way repeated measures analyses of variance as well as non parametric Dunn's analyses, when appropriate. An α level of 0.05 was used for all statistical analyses. *Post hoc* comparisons, when necessary, were conducted using Student-Newman-Keul's (SNK) *post hoc* test.

Immunohistochemical procedures

Tissue preparation and Arc immunochemistry: At the appropriate time for sacrifice subjects were administered a sub-lethal dose of sodium pentobarbital (100mg/kg i.p.) and perfused transcardially with phosphate buffered saline (PBS, pH 7.4) and 4.0% paraformaldehyde. Brains were then removed and post-fixed in 4% paraformaldehyde solution for approximately 18hr at 4°C before being transferred to 30% sucrose PBS solution for at least 48 hours at 4°C, or until the

brains sank to the bottom of the jar. Brains were then frozen and sliced into free-floating coronal sections of 40-µm thickness using a cryostat. Starting at the most rostral portion of the hippocampus, every third section was taken and stored in PBS until immunohistochemical processing approximately 24hr later. Twelve slices per subject were chosen on the basis of uniformity between subjects and consisted of 6 dorsal hippocampal sections (-2.76 to -4.36 from bregma) and 6 ventral hippocampal sections (-4.86 to -5.88) for each subject.

Day one of immunohistochemistry consisted of washing slices in PBS for 3 x 10 min, blocking in PBS containing 1% bovine serum albumin (BSA), 0.1% Triton-X for 1 h, and incubating 18-24hr at 4°C in anti-Arc antibody (Cruz Arc (C-7) sc-17839 mouse mono-IgG2A 1:500) in 1%BSA in PBS with 0.1% Triton-X. Day two began with another set of 3 x 10 min washes in PBS followed by incubation in secondary antibody (Vector labs (PK-6102) Vectastain ABC peroxidase kit (Mouse IgG) elite series) in 1%BSA in PBS with 0.1% Triton-X for 1 hr at room temperature. Slices were again washed in PBS for 3 x 10 min, followed by incubation in AB solution for 1 hr and a final 3 x 10 min washes in PBS. Slices were developed in DAB peroxidase substrate for 2-3 min. Slices received a final 3 x 5 min wash in di$H_2$0 before sections were mounted on glass slides, dried, dehydrated with alcohol, and coverslipped using Permount (Fisher Scientific).

Histology: Slices were imaged with a Nikon Eclipse E400 light microscope and captured using ImageJ (NIH) at 4x and 10x magnification levels and saved as jpeg image files. Direct statistical comparisons of absolute cell counts between hippocampal subfields is complicated by cytoarchitectural differences between

those subfields. Specifically, anatomical data indicate that the pyramidal cells within the CA1 subfield of the hippocampus are smaller and more densely packed than pyramidal cells in either CA3 or the dentate gyrus (Pyapali et al., 1998). This higher density of CA1 pyramidal cells thus could lead to a greater number of cells available to express Arc protein compared to CA3, thus making direct comparisons between these subfields potentially misleading. Additionally, there are also differences in the actual size of the subfields within which Arc positive cells were quantified. For example, the size of the ventral CA1 hippocampal subfield is, at some points, twice as large as the dorsal CA1 subfield (Paxinos & Watson, 2007).

To account for these issues, two conversions were used. For one conversion data was normalization of each group to the density of Arc positive cells in home control cage subjects. Normalization to HC levels of Arc expression allows for a more systematic comparison of the specific contributions of Arc protein expression between different behavioral groups and importantly the different subfields. While basal Arc expression is typically quite low (Vazdarjanova & Guzowski 2004), the distribution of baseline Arc protein expression across subfields may not be uniform and our immunohistochemical results support this notion. As such the percent increase in Arc expression relative to that in HC control subjects provides additional information regarding how a particular experience changes Arc expression while controlling for potential differences in baseline expression. By controlling for baseline expression direct comparisons of Arc protein enhancement between CA1 and

CA3 are more meaningful as a higher cell count might be due to higher baseline levels. Importantly, statistical analyses will be reported on both the raw cell counts allowing for a direct comparison of HC Arc expression levels to other experimental groups as well as analysis of percent increase relative to HC subjects for the reasons outlined above. Percent increase will primarily be used when comparing between groups from different experiments in Chapter 4.

An additional conversion of Arc positive cell counts was to divide cell counts by total surface area quantified for each subfield. This conversion is easily made using ImageJ (NIH) by scaling the image and outlining the quantifiable region for a given subfield to determine the size of the area being assessed for the presence of Arc positive cells. Thus with respect to the data presented here, raw Arc positive cell counts obtained using immunohistochemistry were normalized to the size (mm^2) of the area over which Arc expression was quantified. Arc-positive cells within the outlined area were marked and quantified using ImageJ by experimenters blind to the experimental condition of the subject. Individual cell counts per mm^2 were totaled for the 6 dorsal hippocampal sections and the 6 ventral hippocampal sections from each animal, averaged across two or three counters, and then averaged across animals within a group. Importantly, a set of HC subjects' brain tissue was processed each time immunohistochemical analysis was carried out to provide a common and consistent control for each round of tissue staining. For the sake of analysis HC subjects for experiments 2-4 were combined as these animals were handled for the same period of time and had similar levels of Arc protein

expression in all but one subfield. The analysis of HC subject's Arc protein expression across experiments is described in results section of Experiment 2.

Levels of Arc protein expression between and within training conditions were compared. Data was analyzed using separate one- or two-way repeated measures analyses of variance as well as non-parametric Dunn's analyses, when appropriate. An α level of 0.05 was used for all statistical analyses. *Post hoc* comparisons, when necessary, were conducted using Student-Newman-Keul's (SNK) *post hoc* test.

Chapter 3: EXPERIMENTS

Experiment 1. Arc protein expression within discrete subfields of the hippocampus following trace fear conditioning.

Consistent with the evidence reviewed above, a simplified diagram of relevant amygdala and hippocampal projections hypothesized to support trace fear conditioning is shown in Figure E1-1. Briefly, we suggest that trace fear conditioning is likely supported by amygdala projections to CA3 and CA1 of the ventral hippocampus as well as reciprocal connections between ventral CA1, amygdala and dorsal CA3. Based on this proposed circuit we expect trace fear conditioning to enhance Arc expression primarily in ventral hippocampal CA1 and CA3, and to a lesser extent dorsal CA3; we further hypothesize that this enhancement will be specific to animals learning the CS-US association relative to those in a variety of control conditions (see below). Moreover, based on the anatomical projections and functional dissociations described earlier, we expect that simple exposure to a novel context will enhance Arc expression in dorsal CA1 and CA3, with greater levels of Arc expression in dorsal CA3 for animals learning the CS-US association.

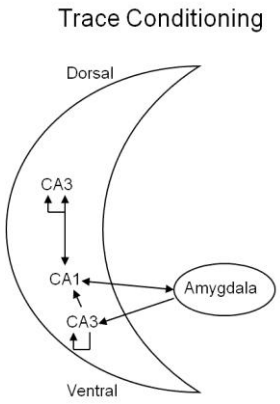

Figure E1-1. A simplified diagram depicting the hippocampal circuits proposed to support the acquisition of trace fear conditioning.

Methods

Variations in procedures to general methods are outlined below.

Subjects. Twenty eight male Sprague-Dawley rats (Harlan, Indianapolis, IN), weighing 250-300g, served as subjects. All subjects were handled for 2 min daily for 5 days prior to the experiment.

Procedure

Behavioral procedures. Auditory trace fear conditioning (**TFC**) (n=5) was conducted using procedures identical to those in previous experiments carried out in the laboratory (Czerniawski et al., 2012; Chia & Otto et al., 2013). In order to determine whether enhancements in Arc expression in TFC animals, if observed, were due specifically to TFC or alternatively to novel context exposure inherent in the TFC procedure, two additional groups of animals were included. The first group (n=5) were trained using an “Extended TFC” (**exTFC**) procedure and received auditory trace fear conditioning identical to the TFC subjects, but

these animals were exposed to the conditioning chamber for two hours immediately prior to trace fear conditioning. This time point was selected because at the time of sacrifice (~3hr 20min) Arc protein induced by exposure to the novel environment would have returned to baseline levels (Lonergan et al., 2010 & Ramirez-Amaya et al. 2005), but not Arc protein induced by trace fear conditioning. Additionally a novel context exposure (**NCE**) (n=5) group was included that was exposed to the same training apparatus used for trace fear conditioning. Animals in this condition were exposed to the training chamber for a period of time yoked to the auditory trace fear conditioning protocol (19min 22sec), but no tones or footshocks were delivered. Additionally a final group of home cage subjects (**HC**) (n = 5) were also sacrificed.

An additional subset of animals were trained using procedures identical to the TFC (n=4) and exTFC (n=4) groups, but were not sacrificed for immunohistochemical analysis. Instead these animals were tested 24hr later in a novel context for fear conditioned to the CS, and then tested again 24hr later (48hr after training) back in the original training chamber to examine levels of contextually-elicited fear. Only the behavioral data for subjects who were both trained and tested are reported in the results section.

Immunohistochemistry and Histology were carried out according to the General Methods section.

Results

Behavioral training and testing

As described previously, a subset of animals from the two groups of subjects trained in auditory trace fear conditioning (TFC: n=4; exTFC: n=4) were tested not only for freezing during training, but additionally during a testing session examining fear conditioned to the tone, and subsequently to fear conditioned to the original training context. The mean (±SEM) percentage of freezing exhibited by TFC and exTFC during training, CS testing, and context testing are presented in Figure E1-2.

Training. The data for Trial 1, prior to the delivery of the first US, were used to assess a ‘‘baseline’’ measure of freezing, and were analyzed separately from those for Trials 2–7. Because freezing for each subject was stable across Trials 2–7 during conditioning, the data for each subject after the first US presentation were averaged into a single value (Trials 2–7). In order to determine if the two hours of pre-exposure to the context in the exTFC group resulted in significantly different levels of freezing during Trial 1 and Trials 2-7 of training relative to that in the TFC group, separate two-way repeated measures ANOVAs were conducted with training condition (TFC, exTFC) as the between subjects factor and trial period (ITI, CS, Trace) as the within subjects factor. For Trial 1 (Figure E1-2a) statistical analyses revealed there was no main effect of condition ($F_{(1,12)}$ = 0.264, p=0.626), no significant main effect of trial period ($F_{(2,12)}$ = 1.762, p=0.213), with no significant interaction between condition and trial period ($F_{(2,12)}$ = 0.238, p=0.792). These analyses suggest there was no effect of context pre-exposure on conditioned responding during trial 1 of training.

For Trials 2-7 (Figure E1-2b) statistical analyses revealed there was no main effect of condition ($F_{(1,12)}$ = 1.423, p=0.278), a significant main effect of trial period ($F_{(2,12)}$ = 20.109, p<0.001), and no significant interaction between condition and trial period ($F_{(2,12)}$ = 1.481, p=0.266). SNK *post hoc* analysis identified significantly higher levels of freezing during the Trace interval compared to during the CS (p<0.001) and ITI (p=0.013) as well as significant differences between the ITI and CS periods, (p=0.005) with the lowest levels of freezing during CS presentation. These analyses suggest there was no effect of context pre-exposure on conditioned responding during trial 2-7 of training.

Tone testing. The data from the first 120 seconds before the first CS presentation (ITI-1) during tone testing reflects “baseline” freezing to the novel testing context and is separated from the other ITI freezing data (FigureE1- 2c). In order to determine if the two hours of pre-exposure to the context in the exTFC group resulted in significantly different levels of freezing to the tone CS during testing relative to that in the TFC group, a two-way repeated measures ANOVA was conducted with training condition (TFC, exTFC) as the between subjects factor and trial period (ITI-1, ITI, CS, Trace) as the within subjects factor. Statistical analyses revealed there was no significant main effect of condition ($F_{(1,18)}$ = 0.201, p=0.669), a significant main effect of trial period ($F_{(3,18)}$ = 1555.42, p<0.001), with no significant interaction between condition and trial period ($F_{(3,18)}$ = 1.54, p=0.238). SNK *post hoc* analysis identified significantly more freezing during the ITI and Trace intervals (p <0.001) than both ITI-1 and CS periods. These analyses demonstrate that while freezing is higher during the trace interval

and ITI than during the CS, there was no effect of context pre-exposure on conditioned responding during tone testing.

Context testing. In order to determine if the two hours of pre-exposure to the context in the exTFC group resulted in significantly different levels of freezing to the conditioning context during context testing relative to that in the TFC group, a two-way repeated measures ANOVA was conducted with training condition (TFC, exTFC) as the between subjects factor and trial period (ITI-1, ITI, CS, Trace) as the within subjects factor. Importantly, during the context test no explicit stimuli are presented; however the data was still analyzed across the same trial periods as the TFC and exTFC groups (i.e. ITI, CS, Trace intervals) in order to provide temporally compatible intervals for comparing across training and testing sessions. Data for one subject in the exTFC group for the context text was lost due to a computer error leaving data from only 7 (TFC n=4; exTFC n=3) subjects for this set of analyses. These data are depicted in Figure E1-2d. Statistical analyses revealed there was no main effect of condition ($F_{(1,10)}$ = 1.405, p=0.289), no main effect of trial period, ($F_{(2,10)}$ =0.1, p<0.906), with no significant interaction between condition and trial period, ($F_{(2,10)}$ = 0.504, p=0.619). Again these analyses suggest there was no effect of context pre-exposure on conditioned responding during context testing, and also no effect of trial period as no stimuli were presented during this session.

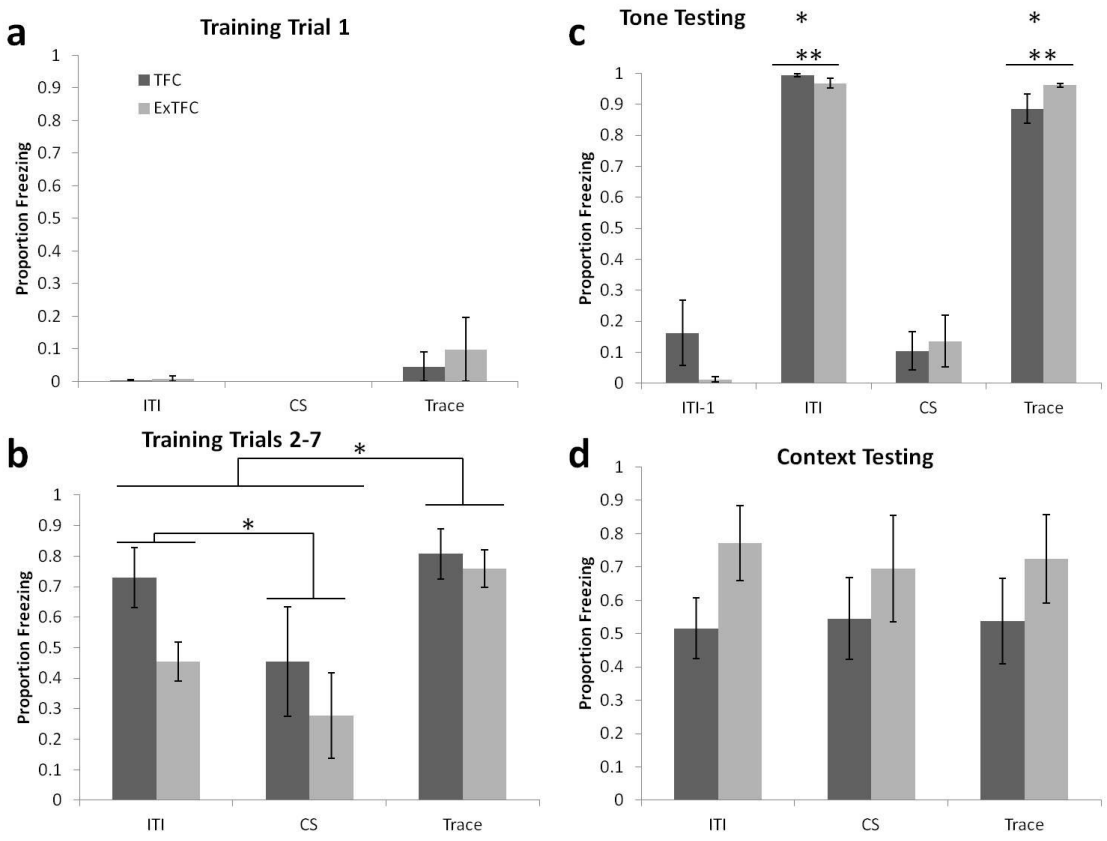

Figure E1-2. Mean +/- SEM portion of session freezing during (a) training trial 1, (b) trials 2-7 with significantly more freezing during the trace interval compared to the CS (p=0,001) and the ITI (p=0.013) as well as during ITI compared to CS (p=0.005), (c) tone testing with significant differences relative to the ITI-1 (*); and CS(**) (p<0.001), and (d) context testing were no significant differences were identified.

Immunohistochemical examination of the regional patterns of Arc expression

Hippocampal neuronal cells positive for Arc protein expression were quantified across the septotemporal (Figure E1-3a) and transverse (Figure E1-3b) axes of both dorsal and ventral hippocampus. Statistical analyses are reported for both

raw Arc positive cell counts/mm^2 as well as percent increase in Arc protein relative to HC subjects.

For one HC animal the perfusion procedure resulted in hippocampal slices which were not appropriately stained with DAB peroxidase. This animal was excluded from the analyses. Two other animals demonstrated levels of Arc expression in ventral CA1 which were determined to be outliers by Dixon's outlier test: one HC subject ($Z_{(3)}$ =1.48, p<0.05), and one exTFC subject ($Z_{(4)}$ = 1.715, p<0.05). These two animals were also excluded from statistical analyses; final sample sizes for were TFC: n=5; exTFC: n=4, NCE: n=5; HC: n=3.

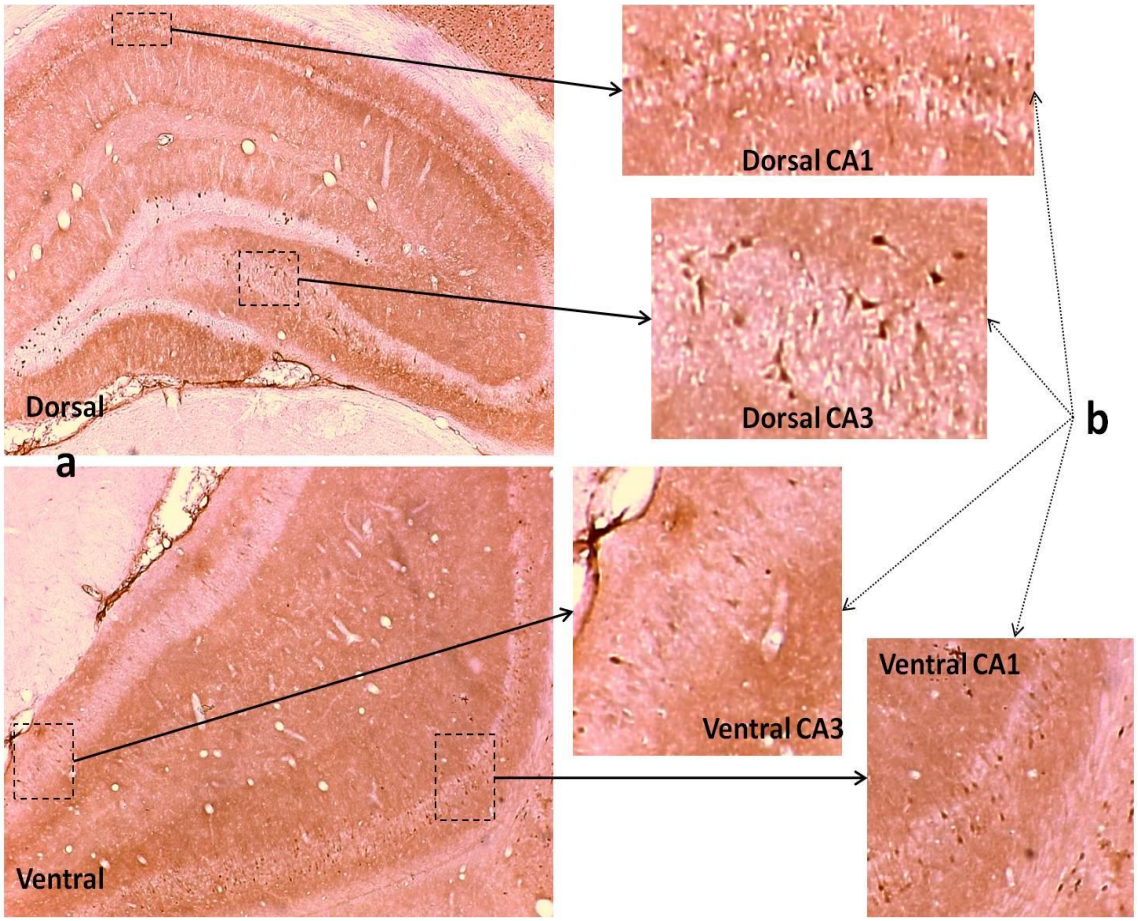

Figure E1-3. Representative Arc positive protein expression in (a) dorsal (top left) and ventral (bottom left) hippocampus (septotemporal axis) and within (b) CA1 and CA3 subfields (transverse axis) for a subject trained in trace fear conditioning.

Arc positive cells per mm^2 in dorsal and ventral hippocampal subfields CA1 & CA3 compared to HC control subjects. In order to determine if different kinds of training differentially affected Arc protein expression, a two-way repeated measure ANOVA was conducted on dorsal hippocampus immunohistochemical data (Figure E1-4a) with training condition (TFC, exTFC, NCE, HC) as the between subjects factor and hippocampal subfield (CA1, CA3) as the within subjects factor. Statistical analyses revealed there was no main effect of condition ($F_{(3,13)}$ = 0.826, p=0.503), a significant main effect of subfield, ($F_{(1,13)}$ =8.033, p=0.014), with no significant interaction between condition and subfield, ($F_{(3,13)}$ = 0.619, p=0.615). Theses analyses suggest there is no effect of training on Arc protein expression and greater levels in dorsal CA1 are independent of training type.

A separate two-way repeated measure ANOVA was conducted on ventral hippocampus immunohistochemical data (Figure E1-4b) with training condition (TFC, exTFC, NCE, HC) as the between subjects factor and hippocampal subfield (CA1, CA3) as the within subjects factor. Statistical analyses revealed there was a significant main effect of condition ($F_{(3,13)}$ = 4.337, p=0.025), a significant main effect of subfield, ($F_{(1,13)}$ =13.41, p=0.003), with no significant interaction between condition and subfield, ($F_{(3,13)}$ = 1.254, p=0.331). SNK *post hoc* analysis identified HC subjects' mean Arc expression to be significantly lower

than TFC subjects (p=0.015) and approaches significance for NCE subjects (p=0.054). Contrary to the dorsal hippocampus theses analyses suggest significant enhancements in Arc protein in the ventral hippocampal subfields for subjects trained in TFC compared to HC controls.

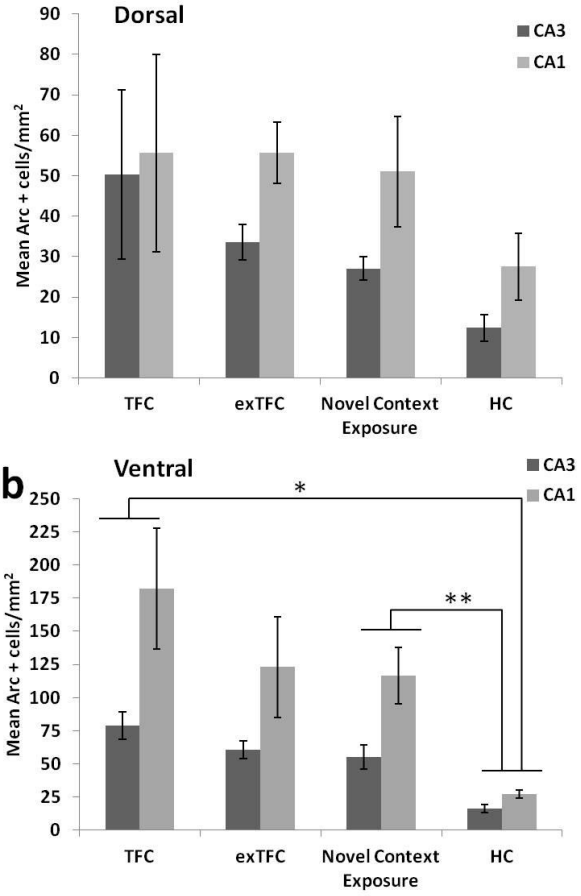

Figure E1-4. Arc positive cells counts in CA3 and CA1 subfields in dorsal and ventral hippocampus. (a) Mean ± SEM Arc positive cells per mm^2 in the dorsal hippocampus. (b) Mean ± SEM Arc positive cells per mm^2 in the ventral hippocampus with significantly enhanced Arc protein for subjects trained in TFC (p=0.015)(*) and near significantly enhanced for Novel context exposure subjects (p=0.054)(**).

Percent increase in Arc positive cells per mm^2 in dorsal and ventral hippocampal subfields CA1 & CA3 relative to HC control subjects. In order to correct for

differences in cell density and compare different training conditions, but also directly compare CA1 vs CA3 subfield enhancement, cell counts were transformed into percent increase relative to HC subjects. In dorsal hippocampus (Figure E1-5a) a two-way repeated measures ANOVA with training condition (TFC, exTFC, NCE) as the between subjects factor and hippocampal subfield (CA1, CA3) as the within subjects factor revealed no significant main effect of condition ($F_{(2,11)}$ = 0.437, p=0.656), a significant main effect of hippocampal subfield ($F_{(1,11)}$ = 6.187, p=0.03), and no significant interaction between training condition and hippocampal subfield ($F_{(2,11)}$ = 1.717, p=0.224). Theses analyses again suggest Arc protein expression is not affected by training type, however, after controlling for baseline levels of expression, Arc protein enhancement is significantly greater in CA3 compared to CA1, opposite of the enhancement pattern reported above prior to transformation. While not statistically significant there is a trend toward even greater CA3 enhancement for TFC subjects.

In ventral hippocampus (Figure E1-5b) a two-way repeated measures ANOVA with training condition (TFC, exTFC, NCE) as the between subjects factor and hippocampal subfield (CA1, CA3) as the within subjects factor demonstrated no significant main effect of condition ($F_{(2,11)}$ = 1.623, p=0.241), no significant main effect of hippocampal subfield ($F_{(1,11)}$ = 2.972, p=0.113), and no significant interaction between training condition and hippocampal subfield ($F_{(2,11)}$, = 0.247, p=0.785). Theses analyses suggest there is no effect of training on Arc protein expression as well as no differences between ventral CA1 and CA3 subfields.

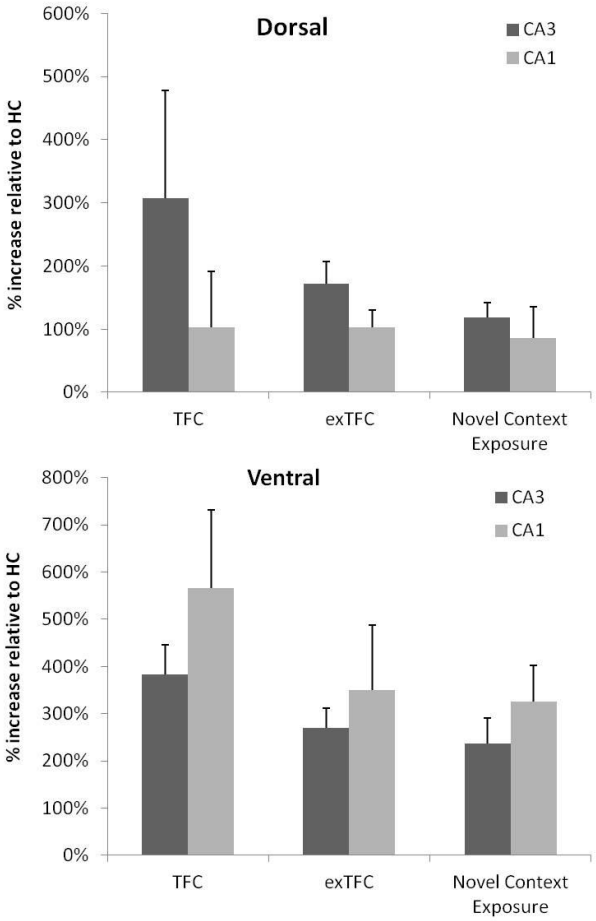

Figure E1-5. Mean ± SEM percent increase in Arc positive cells relative to HC subjects per mm^2 in the (a) dorsal hippocampus and (b) ventral hippocampus.

Discussion

Previously published data from our laboratory demonstrated that Arc expression within the hippocampus is significantly enhanced in animals trained in trace fear conditioning relative to HC control subjects as determined by both Western Blotting protein analysis and RT-PCR analysis of mRNA on fresh hippocampal tissue (Czerniawski et al., 2011; Chia & Otto, 2013). The present results extend these previous findings in a variety of important ways. The current

data also include additional behavioral groups designed to better characterize the specific aspects of the trace fear conditioning paradigm which result in enhancement of Arc protein expression.

Behavioral measures of freezing did not differ between TFC and exTFC subjects

We expected that extended pre-exposure to the training context would reduce context-shock associations and, in turn, contextually-elicited fear during later testing. In contrast to our predictions, behavioral measures of contextually-elicited freezing did not differ between animals trained in TFC compare to exTFC (Figure E1-2d). The lack of a significant behavioral effect of context pre-exposure for exTFC subjects may have contributed to the lack of difference in levels of Arc expression between exTFC, TFC, and Novel Context Exposure subjects (see below).

Arc protein expression

Animals trained in trace fear conditioning (TFC, exTFC) and animals receiving novel context exposure demonstrated substantial increases in Arc expression in both the dorsal and ventral hippocampus compared to HC subjects (Figure E1-4). Within the dorsal hippocampus Arc protein expression was significantly different between CA1 and CA3 both when compared directly with HC subjects (Figure E1-4a) and when considered relative to HC subjects (Figure E1-5a). Arc protein expression, across behavioral groups, was greater in CA1 relative to CA3 in dorsal hippocampus (Figure E1-4a). However when baseline

differences are controlled for (see general methods), trace fear conditioning (TFC, exTFC groups) and novel context exposure (NCE) enhances Arc expression preferentially in dorsal CA3 compared to CA1 (Figure E1-5a), partially supporting our initial hypothesis, and while CA3 was not significantly enhanced relative to HC subjects prior to transformation, there was a trend. Within the ventral hippocampus Arc protein expression was also significantly different between CA1 and CA3 across behavioral groups (Figure E1-4b). However, when baseline differences are controlled for differences between CA1 and CA3 are no longer observed. However, unlike the dorsal hippocampus only showing a trend toward enhancement in CA3, both ventral CA1 and CA3 subfields show significant enhancement relative to HC subjects (Figure E1-5b). As such, trace fear conditioning (TFC, exTFC) and novel context exposure (NCE) enhanced Arc protein expression in the ventral hippocampus in both CA3 and CA1 compared to HC control subjects consistent with our hypothesized circuit supporting trace fear conditioning (Figure E1-1).

Dorsal hippocampus Arc protein expression

Trends in the regional distribution of Arc positive cell counts within individual subfields are generally consistent with our initial hypotheses. The current results are consistent with anatomical evidence suggesting dissociable functional roles of different regions of the hippocampus that likely support trace fear conditioning. More specifically, afferent projections to dorsal hippocampus CA3 from ventral CA1 provides the major pathway for amygdala activity to reach the dorsal hippocampus, and is required to support contextual fear conditioning (Hunsaker

& Kesner, 2008). This ultimately suggests that plasticity and Arc expression within dorsal CA3, and not CA1, may reflect some aspects of trace fear conditioning, particularly context-shock associations, as trained animals also demonstrate freezing to the training context (Figure E1-2d). This supports and extends previous data from our laboratory on the role of plasticity and Arc expression in the dorsal hippocampus for contextual fear conditioning (Czerniawski et al. 2012). While there has yet to be a systematic investigation of the relative roles of Arc expression in the dorsal hippocampal subfields in the acquisition of trace fear conditioning, the current results are consistent with our data in that Arc expression in CA3, but not CA1, of the dorsal hippocampus, while not significant, tended to be greater in subjects trained in trace fear conditioning (TFC, exTFC) than HC controls (Figure E1-4a). These results are consistent with evidence supporting the differential role of dorsal CA3 versus CA1 in contextual fear (Ramamoorthi et al., 2011). Moreover, the significant difference in Arc expression in dorsal CA3 versus CA1, after controlling for baseline Arc expression in HC subjects, overall further supports the specific role of dorsal CA3 in modulating contextually elicited fear (TFC, exTFC) (Figure 2d). While these trends are present in TFC and exTFC trained subjects, and not Novel Context Exposure subjects, levels of Arc expression did not differ significantly between groups. The lack of a significant effect of training condition precluded a more in-depth statistical comparison of CA3 versus CA1 across different animal groups.

Trends in the regional distribution of Arc positive cell counts between different behavioral groups are generally consistent with our initial hypotheses. Within the dorsal hippocampus, Arc protein expression was expected to be preferentially enhanced in CA3 relative to CA1 for subjects trained in trace fear conditioning, with a greater enhancement for subjects who experience higher levels of contextually-elicited fear. The current data partially support these hypothesized differences in Arc protein expression between different behavioral groups. After controlling for baseline expression, Arc enhancement in the dorsal CA3 region in was greater in TFC subjects relative to exTFC subjects (Figure E1-5a), although this trend did not reach statistical significance. This trend supports our hypothesis, as TFC subjects were exposed to both tone-shock paring and a novel context during training. For exTFC subjects the present data are then partially consistent with previous observations in that both dorsal CA3 and CA1 have previously been identified to demonstrate an increase in Arc expression due to novel context exposure (Ramirez-Amaya et al. 2005). This suggests that pre-exposure to the training context for exTFC subjects was sufficient to drive down Arc protein expression within dorsal CA3 but was not sufficient to reduce expression in dorsal CA1 relative to TFC subjects. The current data regarding dorsal CA1 expression suggests similar effects of trace fear conditioning (TFC, exTFC) and novel context exposure groups as there was no apparent trend toward decreasing expression as seen in dorsal CA3 reported above. While these findings are contrary to other data which show enhanced Arc expression in both dorsal CA3 and CA1 Arc expression following novel context exposure

(Ramirez-Amaya et al. 2005; Lonergan et al., 2010), these data do support the differential role of dorsal CA3 versus CA1 in mediating contextual fear mentioned above. This notion is further supported in that Novel Context Exposure subjects do not show a similar trend of greater Arc expression in CA3 versus CA1 as these subjects did not receive fear conditioning and hence would not exhibit contextually elicited fear.

Ventral hippocampus Arc protein expression

Regional distributions of Arc positive cell counts within individual subfields are generally consistent with our initial hypotheses. TFC subjects exhibited a significant increase in Arc protein expression relative to HC subjects, and differences between Novel Context Exposure subjects and HC subjects approach significance (p=0.054) (Figure E1-4b). Hypothesized differences between TFC, exTFC, and Novel Context Exposure subjects are only partially supported when Arc protein expression is compared across subfields of interest (CA1, CA3) and controlling for baseline Arc expression (Figure E1-5b). As predicted there were no significant differences in Arc protein expression between the TFC group and the exTFC groups as both of these groups were trained in trace fear conditioning. Yet, there was also no significant difference between Novel Context Exposure subjects and subjects trained in trace fear conditioning (TFC, exTFC), which was unexpected as these animals were not trained in our fear conditioning protocol and as such amygdala input to the ventral hippocampus should have been minimized. While these effects were inconsistent with our initial predictions, this is the only study to date which has

investigated Arc protein expression in the ventral hippocampus after novel context exposure; the Arc expression data regarding novel context exposure will be discussed in more detail below.

While prior data from our laboratory has previously demonstrated deficits in trace fear conditioning when the ventral hippocampus is infused with either Arc ODNs or APV prior to training (Czerniawski et al., 2011; Czerniawski et al., 2012; Chia & Otto 2013), as well as following ventral hippocampus excitotoxic lesions (Czerniawski et al., 2009), there has yet to be a systematic investigation of the role of the transverse ventral hippocampal subfields in the acquisition of trace fear conditioning. Anatomical evidence described above suggests that both CA1 and CA3 receive amygdala afferent input, and consistent with the well-established role of the amygdala in fear conditioning (LeDoux, 1995), Arc expression in both CA1 and CA3 of ventral hippocampus in animals trained in trace fear conditioning (TFC, exTFC) exhibit a substantial increase relative to HC controls (Figure E1-4b). More specifically, higher levels of Arc expression were observed in ventral CA1 compared to CA3 for subjects train in trace fear conditioning (TFC, exTFC). Though not statistically significant, these trends are also largely consistent with anatomical evidence identifying reciprocal connectivity between ventral CA1, amygdala and dorsal CA3 (Figure E1-1), while the same reciprocal connections are not present in ventral CA3 and are consistent with the current experiment demonstrating higher levels of Arc expression in ventral CA1 compared to CA3. Contrary to our hypothesis, levels of Arc protein expression in exTFC subjects were similar to Novel Context

Exposure subjects (Figure E1-5b). Elevated levels of Arc expression for Novel Context Exposure subjects will be discussed in more detail below.

Regional distributions of Arc positive cell counts between different behavioral groups are generally consistent with our initial hypotheses. Ventral CA1 and CA3 Arc expression was greater in TFC trained subjects compared to exTFC subjects (Figure E1-5b) but more so in CA1 than CA3. This relationship was predicted based on our assumption of a reduction in contextually-elicited fear for exTFC animals due to their pre-exposure to the training context prior to conditioning. Reductions in contextually-elicited fear were expected to be mediated by reduced amygdala-hippocampal communication, specifically in ventral CA1 (and not ventral CA3) as reciprocal communication between ventral CA1 and dorsal CA3 (Figure E1-1) is implicated in contextual fear. Yet the lack of a significant behavioral effect of pre-exposure (described above) may account for the lack of significant differences between TFC and exTFC animals' Arc expression. However, there was also a lack of significant differences in CA3 and CA1 for subjects trained in trace fear conditioning (TFC, exTFC) relative to Novel Context Exposure subjects. NCE subjects were hypothesized to have markedly less Arc protein expression in both ventral hippocampal subfields due to the lack of explicit fear conditioning in that animal group (Figure E1-5b) however this was not observed. Overall, differences in Arc expression in CA1 relative to CA3 are partially consistent with anatomical evidence and training differences for subjects trained in fear conditioning (TFC, exTFC), however the high levels of Arc expression in the Novel Context Group was unexpected, specifically in ventral

CA1, and may be due to previously unidentified communication between the ventral hippocampus and amygdala during novel context exposure. This will be discussed in more detail below.

Arc expression induced by novel context exposure is similar to that induced by trace fear conditioning

To our knowledge the present study is the first to examine the effect of novel context exposure on hippocampal Arc expression in both the dorsal and ventral hippocampus across subfields. While others have identified enhancement in dorsal CA1 and CA3 following novel context exposure (Ramirez-Amaya et al., 2005), our results suggest both dorsal and ventral hippocampal CA1 and CA3 show a substantial, though not always statistically significant, increase relative to HC subjects.

The similar level of Arc expression within the ventral hippocampus of Novel Context Exposure subjects compared to subjects trained in fear conditioning (Figure E1-5b) was unexpected. It is possible that within the Novel Context Exposure group, elevated levels of Arc expression in ventral hippocampus may serve a modulatory function in the dorsal hippocampus. Arc RNA transcription in the dorsal hippocampus has been implicated in location-specific firing of CA3 and CA1 hippocampal neurons, which in turn has been related to the establishment of hippocampal place fields (Bramham et al., 2008; Guzowski et al, 1999). Importantly, Arc mRNA translation can be subject to modulation (McIntyre et al., 2005) via posttranscriptional regulation by amygdala-

dependent neuromodulatory processes (Bramham et al., 2008). This suggests a role for amygdala connections in mediating dorsal, and perhaps ventral, hippocampal Arc protein expression seen in Novel Context Exposure groups. Yet, the involvement of the amygdala in modulating Arc expression in the hippocampus, in the absence of explicit fear conditioning, is unclear. There is evidence, however, to suggest a role of the amygdala in responding to novel objects and contexts (Moses et al., 2002). Specifically, rats with amygdala lesions have shown attenuated neophobic responses to novel food stimuli (Burns et al., 1996; Dunn & Everitt, 1988; Rolls & Rolls, 1973; Sutherland & McDonald, 1990). This evidence, coupled with general neophobia observed in rats within a novel context, may suggest a role of amygdala modification of hippocampal Arc expression in the absence of explicit trace fear conditioning or other hippocampal-dependent aversive learning experiences. As such, given the anatomical evidence identifying the ventral hippocampus as the primary pathway by which amygdala inputs would reach the dorsal hippocampus, Arc expression within the ventral hippocampus may modulate relevant dorsal hippocampal activity. This would include the establishment of dorsal hippocampal place fields in novel environments, as well as the possibility of a more general preparation for additional amygdala afferent input to modulate more explicit aversive learning events and behavioral change within a potentially aversive/fearful novel context. Hence Arc protein related plasticity in the ventral hippocampus could occur in the absence of explicit fear conditioning. Yet it is unclear, based on the present data,

which set of connections between the ventral hippocampus and amygdala account for observed Arc expression patterns.

In order to further address the extent to which novel context exposure contributed specifically to the patterns of results observed here, future studies will include a group of animals that is repeatedly exposed to the novel training context prior to training and immunohistochemical analysis. Within these behavioral groups pre-exposure to the conditioning chamber is expected to produce contextual latent inhibition to the training context relative to non-exposed behavioral groups. This will ideally bias Arc protein expression toward hippocampal activity supporting CS-US learning relative to context-US learning. With respect to Arc protein enhancement, we expect this pre-exposure to be sufficient to prevent novel context induced Arc expression.

Behavioral measures of freezing reduced during CS presentation

Consistent with previous observations from our lab (Czerniawski et al., 2011, Chia & Otto, 2013), the freezing response during CS presentation was significantly lower than during other trial periods during both training (Figure E1-3b) and tone testing (Figure E1-3c) but not context testing (Figure E1-3d). One potential account for this is based on previous research demonstrating less conditioned responding during a CS presentation for subjects learning a trace conditioning task compared to delay conditioning task (Davitz et al., 1957; Kamin 1954 & 1961; Mowrer and Lamoreaux, 1951). This difference in responding is generally attributed to differences in the strength of the learned CS-US

association. While this is a possibility, freezing during the other trial periods (Trace, ITI) are at near ceiling levels (Figure E1-3c), making it difficult to suggest that the task itself elicits less conditioned responding. Another possible account for lower levels of freezing during CS presentation is that the animals are learning a general temporal pattern of freezing and mobility which is consistent with the time of the delivery of the US across trials, irrespective of other stimuli in the environment (CS, context) (Gallistel & Gibbon, 2000). However, we do not see this same pattern of responding when the CS is not presented during CR testing back in the original training context where this temporal pattern was initially experienced. Another alternative interpretation is that animals are acquiring a CS-no-shock association. In this sense the CS may act as a kind of "safety" signal for a period of time for which the US will not occur. With respect to the current experiment, adopting one interpretation over another is irrelevant for our purposes and predictions, but it is mentioned here as it will become an important observation in the discussion of the experiments to follow.

Conclusions

Arc protein was enhancements for subjects trained in TFC in ventral CA1 and CA3 with trends toward enhancement in dorsal CA3 compared to HC control subjects. However the general lack of statistical differences in Arc expression between the TFC, exTFC and Novel context exposure groups could reflect the possibility that increases in Arc protein expression in both the dorsal and ventral hippocampus were due to novel context exposure, and that Arc expression within these regions may not itself be unique to the acquisition of trace fear

conditioning. Given the potential role of the amygdala and the effects of novel context exposure on Arc protein expression in the ventral hippocampus outlined above, the specific way in which blocking Arc protein impairs learning in trace fear conditioning (Czerniawski et al., 2011) may be more complicated. Instead of Arc ODNs directly blocking explicit CS-US learning-related plasticity, perhaps preventing Arc expression induced by novel environment exposure, in either the dorsal or ventral hippocampus, interferes with the animal's ability to learn context associations within that novel context. If an animal cannot learn about the context-CS associations, or context-US association due to inhibited Arc expression, then the specific acquisition of the CS-US association may be compromised as well. While this notion is speculative, it highlights the importance of and need for sophisticated behavioral controls to identify how compromising neuronal function leads to changes in behavior.

Experiment 2. Prior exposure to a context prevents novel context induced Arc protein expression

To our knowledge the previous experiment is the first documentation of Arc protein enhancement in ventral hippocampus following novel context exposure. This observation, while unique, poses issues which interfere with our ability to address our initial hypothesis seeking to identify hippocampal subfields uniquely involved in trace fear conditioning. The most unexpected outcome of the prior experiment was that the enhancement in Arc protein in the dorsal and ventral hippocampus for animals exposed to a novel environment was statistically undifferentiated from both TFC and exTFC subjects. Arc protein enhancement following novel context exposure is a well reported phenomenon in dorsal hippocampus CA1 and CA3 (Ramirez-Amaya et al., 2005; Vazdajanova et al., 2006) as Arc expression is implicated, in part, in place cell remapping; hence the formation and stabilization of place cell fields is likely one of the drivers of Arc expression during novel context exposure (Vazdajanova and Guzowski, 2004; Guzowski, 1999). Hippocampal “place cells” have been identified in the ventral hippocampus, but they are reduced in number and spatial selectivity (Jung et al., 1994).

The first issue is that the Arc protein enhancement in trained animals in the previous experiment cannot be dissociated from enhancement due to novel context exposure. Our lab and others have pre-exposed animals to the conditioning context prior to training, providing sufficient time for induced Arc protein to return to baseline levels (Czerniawski et al., 2011; Ramirez-Amaya et

al., 2005; but see Lonergan et al. 2010). However, following this pre-exposure and initial wave of Arc protein, subjects are then exposed to a behavioral procedure expected to further induce Arc protein enhancement. This task induced expression presumably occurs after the initial wave of Arc enhancement due to novel context exposure has waned. Though this poses a potential problem as any long lasting structural modifications produced by this initial wave of Arc protein (Link et al., 1995; Lyford et al., 1995; Steward et al., 1998; Shepherd & Bear, 2011) may differentially interact with Arc protein induced by the behavioral task itself, fear conditioning or otherwise. This is particularly relevant with respect to the prior experiment in which training occurred at a time point in which Arc protein enhancement due to context exposure would have recently returned to baseline levels (Lonergan et al., 2010). The second issue is that we have no way of knowing the conditions under which a context transitions from one which is novel, and induces Arc protein expression, to one which is familiar and does not enhance Arc expression (similar to the animals' home cage). Thus we sought to identify a set of parameters by which exposure to the conditioning context produces a level of Arc protein expression undifferentiated from that of HC subjects.

Latent inhibition (LI) refers to a reduction in conditioned responding to a conditioned stimulus to which an organism has had prior exposure compared to another, unfamiliar CS. Thus, the LI procedure provides a means of biasing the "amount" of learning between different CSs. Barot et al., (2009) took advantage of this procedure to examine Arc mRNA following contextual fear conditioning to

identify cell populations in the dorsal hippocampus and amygdala with convergent CS-US information. Utilizing a contextual LI procedure as a “no learning” control, some subjects were pre-exposed to the conditioning context for 10 days prior to contextual fear conditioning. While not the main focus of the experiment, Barot et al., found that Arc mRNA expression in LI subjects was reduced in various subregions in the hippocampus and amygdala compared to untrained control subjects exposed to the novel conditioning context (2009). While there was not a “no-context exposure” home cage control group by which to compare, these data suggest that the pre-exposure period used by Barot et al., in the LI group reduced Arc mRNA induced by novel context exposure. Though the specific experiential events which modulate Arc mRNA transcription differently than Arc protein translation in vivo are not well characterized, it can be assumed that if mRNA is reduced then the downstream protein product may also be similarly affected. For example Ramirez-Amaya et al., (2005) have demonstrated relatively linear temporal dynamics between Arc mRNA transcription and later protein translation, showing that enhancement in mRNA within a given subfield is closely followed by an enhancement in protein within that same subfield.

For Barot et al. (2009), pre-exposing subjects to a conditioning context also produced a reduction in mobility during training, suggesting familiarity with the context as stimulus/context novelty is often measured by examining the exploratory behavior the stimulus elicits (Dere et al., 2005). Based on this evidence, we expect that if a context is familiar, and does not induce mobility,

then Arc protein will not be meaningfully enhanced above that of HC subjects. With that in mind, the current experiment adopted a paradigm similar to that used by Barot et al., (2009) to directly compare behavioral mobility and Arc protein expression following exposure to a novel context vs. a context to which and animal has been repeatedly exposed (i.e. a "familiar" context). The context pre-exposure in the current experiment is different from the pre-exposure for exTFC subjects in Experiment 1 because in the present study subjects were repeatedly exposed to the conditioning context across multiple days as opposed to the single pre-exposure session of 2hr used in Experiment 1. Additionally, subjects in the current experiment will not receive fear conditioning and are only exposed to the novel or familiar context prior to sacrifice for immunohistochemical analyses. Protein expression and exploratory behavior is expected to be reduced in animals for which the context is familiar compared to one which is novel.

Methods

Variations in procedures to general methods are outlined below.

Subjects. 13 male Sprague-Dawley rats (Harlan, Indianapolis, IN), weighing 250-300g, served as subjects.

Procedure

Context pre-exposure. Context pre-exposure took place over 10 days and consisted of removing animals from their home cages, handling them for 2 minutes, placing them in an empty transfer cage and bringing them into the room with the conditioning chambers (described in general methods) and placing them

into the apparatus for approximately 20 minutes. During this time the houselight was on and the chamber illuminated. No other stimuli were presented during pre-exposure. After 20 minutes the subject was removed and returned to his home cage. Animals in this experiment were not trained (NT) in a fear conditioning task and were only exposed to the conditioning context and thus for these animals the context was familiar (**F-NT**, n=5). A second group of animals, with identical daily handling and transporting within transport cages, were not pre-exposed to the conditioning chamber and thus for these animals the context was novel (**N-NT**, n=5). Additionally, a final group of home cage control subjects (**HC**) (n = 3) were also sacrificed.

Context exposure. Context exposure occurred after the pre-exposure period described above for both F-NT and N-NT groups. Animals were placed into their respective context for 19’22” and no other stimuli were presented. During context exposure mobility behavior was recorded each second by the motion sensor attached to the top of the apparatus. After context exposure animals were removed and returned to their home cage for one hour prior to sacrifice and tissue processing for immunohistochemical analysis of Arc protein expression (described in General Methods).

Results

Behavioral immobility

Immobility was measured for F-NT and N-NT groups during the final context exposure prior to sacrifice for immunohistochemistry. Immobility data was

converted into proportion of each minute immobile for 19’22” (Figure E2-1) for visual analysis of changes in immobility across time. After observing the different trends in responding across time, immobility was averaged across the first and last five minutes of context exposure for the sake of statistical analysis (Figure E2-2). Levels of mobility are commonly used to assess an animal’s familiarity with a context, with decreasing mobility (or increased ***im***mobility, as measured here) and behavioral exploration interpreted as behavioral evidence of familiarity with context/objects (Dere et al., 2005). Two subjects’ behavioral data, one from each group, were unavailable due to errors in the motion detector in one of the conditioning chambers. Final sample sizes for behavior data were n=4 from both F-NT and N-NT.

Statistical analysis of the affects of pre-exposure to the conditioning context was conducted on the data averaged across minutes presented in Figure E2-2. Pre-exposure to the conditioning context was expected to significantly increase immobility as determined by a two-way repeated measures ANOVA with context exposure (F-NT vs. N-NT) as the between subjects factor and time (first 5 minutes vs. last 5 minutes) as the within subjects factor. Statistical analyses of immobility revealed a significant main effect of pre-exposure ($F_{(1,15)} = 71.28$, $p<0.001$), a significant main effect of time ($F_{(1,15)} = 95.00$, $p<0.001$), with a significant interaction between pre-exposure and time ($F_{(1,15)} = 81.47$, $p<0.001$). SNK *post hoc* analysis identified mean time spent immobile to be significantly different between F-NT and N-NT subjects during the last 5 minutes of testing ($p<0.001$) but not during the first 5 minutes ($p=0.464$). These results replicate the

contextual latent inhibition effect documented by Barot et al., (2009) and support our hypothesis that pre-exposure to a context will result in more immobility compared to non-pre-exposed subjects for which the context is novel.

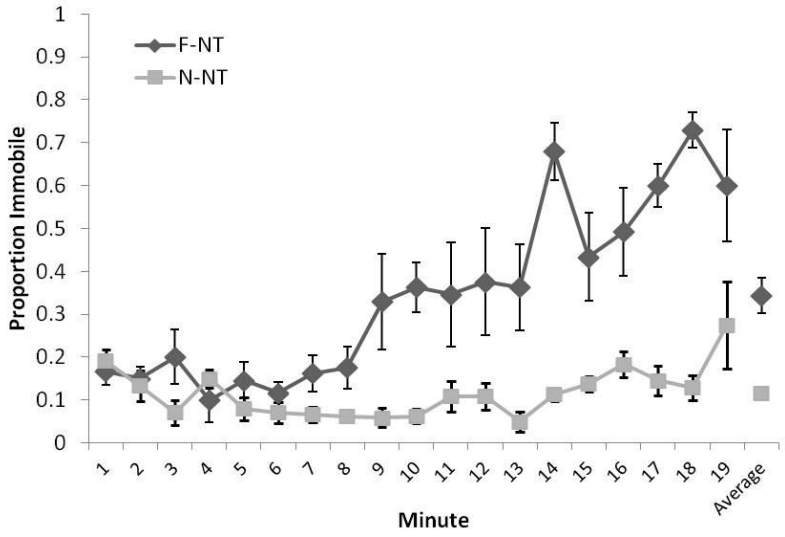

Figure E2-1: Average (+/-SEM) proportion of each minute immobile during exposure to a familiar (F-NT) or novel context (N-NT)

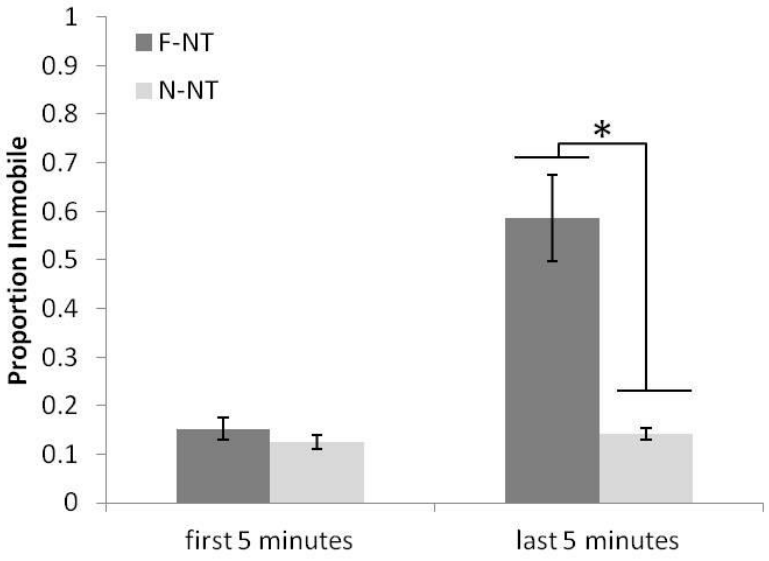

Figure E2-2: Average (+/-SEM) proportion immobile during the first 5 minutes and last five minutes during exposure to a familiar or novel context. SNK *post hoc* analysis identified mean time spend immobile to be significantly different

between N-NT and F-NT subjects during the last 5 minutes of testing (p<0.001) but not during the first 5 minutes (p=0.464).

Immunohistochemical examination of regional patterns of Arc expression

Hippocampal neuronal cells positive for Arc protein expression were quantified across the septotemporal and transverse axes of both dorsal and ventral hippocampus. Statistical analyses are reported for Arc positive cell counts/mm^2. Before analyzing Arc protein expression within the current experiment, groups which were similar across experiments were compared (home cage subjects, Novel context exposure subjects).

Comparison of Arc positive cells per mm^2 in home cage subjects across experiments

In order to provide a common baseline level of Arc expression from which experience/conditioning dependent enhancement can be assessed, HC subjects were sacrificed across all experiments (2-4) for each round of immunohistochemical analysis. Groups of HC subjects were compared across experiments to determine if any specific set of HC subjects demonstrated significantly differences within CA3 and CA1 subfields in the dorsal and ventral hippocampus and these data are depicted in (Figure E2-3). Separate two-way repeated measure ANOVA were conducted on dorsal and ventral hippocampus immunohistochemical data for HC subjects across experiments with experiment (2,3,4a,4b) as the between subjects factor and hippocampal subfield (CA1, CA3) as the within subjects factor. Within the dorsal hippocampus (FigureE2-3a)

statistical analyses revealed there was no main effect of experiment ($F_{(3,23)}$ = 3.352, p=0.076), a significant main effect of subfield, ($F_{(1,23)}$ =61.319, p<0.001), with a significant interaction between experiment and subfield, ($F_{(3,23)}$ = 10.288, p=0.004). SNK *post hoc* analysis identified a significant effect of subfield only for Experiment 4a (p<0.001) and Experiment 4b (p<0.001). Significant differences across experiments were only observed in Dorsal CA1 with Experiment 4a and 4b significantly different than Experiment 2 (p=0.004, p=0.005, respectively) and Experiment 3 (p=0.010, p=0.018, respectively). However, a One way ANOVA comparing dorsal CA1 Arc protein expression across experiments to the average HC value (n=12) (Figure E2-3b) identified no significant differences between groups ($F_{(4,23)}$ = 2.369, p=0.089). Differences in HC subjects Arc expression across 4 groups of HC subjects were only observed in dorsal hippocampus CA1 but no groups were significantly different from the average dorsal CA1 Arc protein expression.

Statistical analyses of Arc protein in the ventral hippocampus (Figure E2-3c) revealed there was no significant main effect of experiment ($F_{(3,23)}$ = 0.132, p=0.939), a significant main effect of subfield, ($F_{(1,23)}$ =20.259, p=0.002), with no significant interaction between experiment and subfield, ($F_{(3,23)}$ = 1.869, p=0.213) (FigureE2-3b). Arc expression in CA1 was greater than CA3 across experiment groups but protein expression was not different between experiments.

While there was some variability in the twelve HC subjects' Arc positive cell counts/mm^2 from Experiments 2-4, across experiments none of the interpretations of Arc enhancement changed when using all twelve HC subjects

compared to just the three HC subjects specific to a given experiment. One exception is within dorsal CA1 following training for DFC subjects which will be discussed more completely within that section of the manuscript. For ease of analysis we decided to combine HC subjects' data across experiments in order to enhance the statistical power of our comparisons and to provide a common baseline by which to compare Arc protein enhancement across experiments and experimental groups (Section 5, 6). This decision is supported by the statistical analyses reported above.

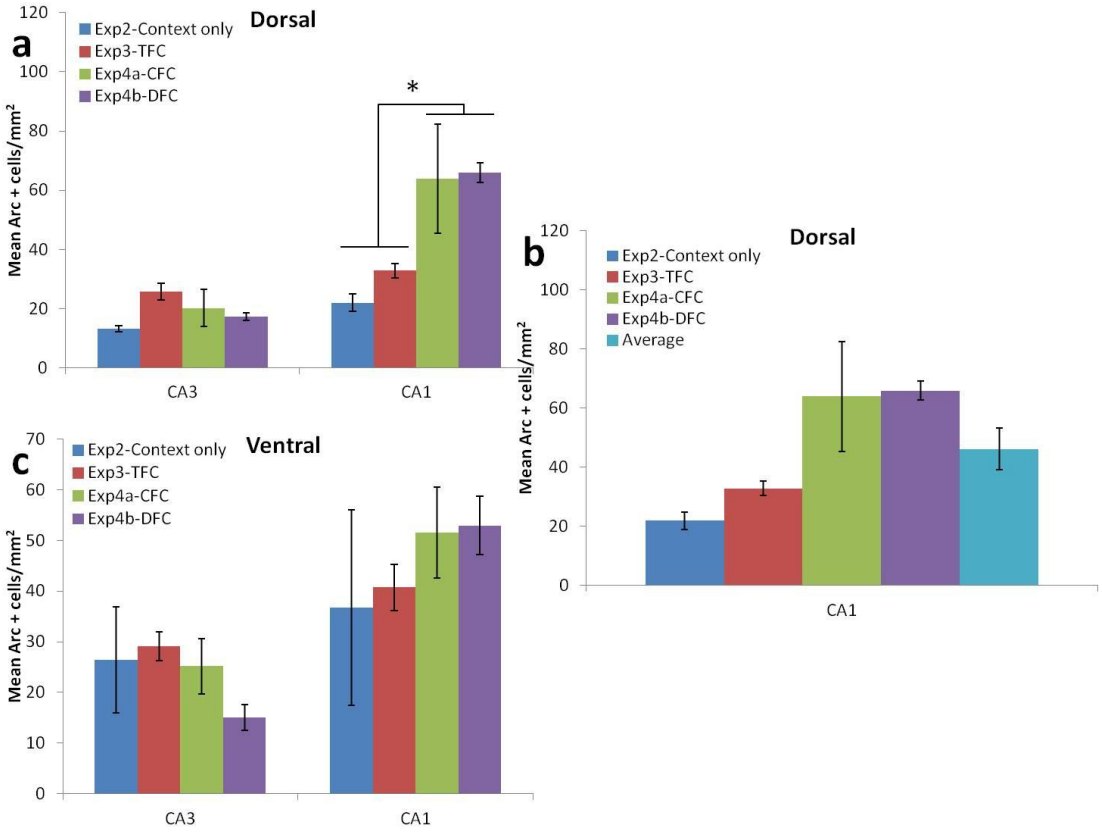

Figure E2-3: Arc positive cells counts in CA3 and CA1 subfields in dorsal and ventral hippocampus for HC subjects across experiments. (a) Mean ± SEM Arc positive cells per mm^2 in the dorsal hippocampus. SNK *post hoc* analysis identified Experiment 4a and 4b to be significantly different than Experiment 2 (p=0.004, p=0.005, respectively) and Experiment 3 (p=0.010, p=0.018,

respectively). Significant differences noted with an asterisk (b) Mean ± SEM Arc positive cells per mm^2 in dorsal CA1 of the dorsal hippocampus for each experiment and the average across experiments. (c) Mean ± SEM Arc positive cells per mm^2 in the ventral hippocampus. Arc protein in ventral CA1 is significantly greater than in CA3 (p=0.002).

Comparison of Arc positive cells per mm^2 in subjects exposed to a novel environment across experiments.

Across Experiment 1 and 2 groups of subjects were exposed to a novel context and differences across experiments were compared in order to see if the pattern of Arc protein enhancement identified in Experiment 1 was replicated in Experiment 2 (FigureE2-4). Two-way repeated measure ANOVA was conducted on dorsal hippocampus immunohistochemical data (FigureE2-4a) for subjects exposed to a novel context in Experiment 1 and 2 with experiment (1, 2) as the between subjects factor and hippocampal subfield (CA1, CA3) as the within subjects factor. Statistical analyses revealed there was no main effect of experiment ($F_{(1,19)}$ = 2.169, p=0.179), no significant main effect of subfield, ($F_{(1,19)}$ =4.206, p=0.074), with no significant interaction between experiment and subfield, ($F_{(1,19)}$ = 1.237, p=0.298). Arc protein expression in the dorsal hippocampus for animals exposed to a novel context was not significantly different across experiments.

A separate two-way repeated measure ANOVA was conducted on ventral hippocampus immunohistochemical data (FigureE2-4b) with experiment (1, 2) as the between subjects factor and hippocampal subfield (CA1, CA3) as the within subjects factor. Statistical analyses revealed there was no significant main effect

of experiment ($F_{(1,19)}$ = 0.0281, p=0.871), a significant main effect of subfield, ($F_{(1,19)}$ =26.666, p<0.001), with no significant interaction between experiment and subfield, ($F_{(1,19)}$ = 1.324, p=0.283). Arc protein expression in the ventral hippocampus for animals exposed to a novel context was not significantly different across Experiment 1 and 2.

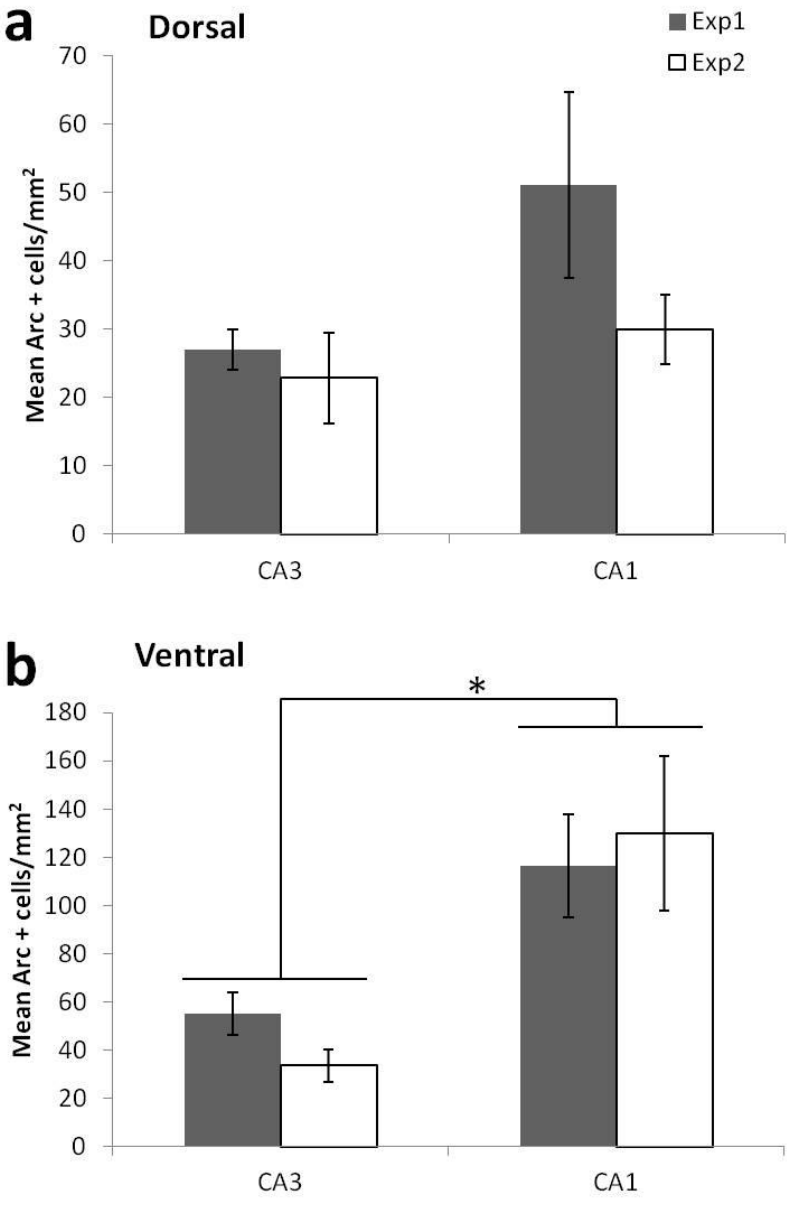

Figure E2-4. Arc positive cells counts in CA3 and CA1 subfields in dorsal and ventral hippocampus for subjects exposed to a novel context in Experiment 1 and 2. (a) Mean ± SEM Arc positive cells per mm^2 in the dorsal hippocampus. (b) Mean ± SEM Arc positive cells per mm^2 in the ventral hippocampus. Statistical analyses revealed a significant main effect of subfield (p<0.001) with more Arc positive cells identified in CA1 compared to CA3 but no difference between experiments.

Arc positive cells per mm^2 for subjects exposed to a novel or familiar context compared to home cage control subjects in dorsal and ventral hippocampal subfields CA1 & CA3

Arc protein expression in CA1 and CA3 for subjects exposed to either a familiar (F-NT) or novel context (N-NT) were compared to HC subjects. Two-way repeated measure ANOVA was conducted on dorsal hippocampus immunohistochemical data with training condition (F-NT, N-NT, HC) as the between subjects factor and hippocampal subfield (CA1, CA3) as the within subjects factor. Statistical analyses revealed there was no main effect of training condition ($F_{(2,43)}$ = 3.485, p=0.051), a significant main effect of subfield, ($F_{(1,43)}$ =10.499, p=0.004), with a significant interaction between condition and subfield, ($F_{(2,43)}$ = 3.759, p=0.042) (Figure E2-5a). SNK *post hoc* analysis identified significant differences in subfield only for HC subjects. Across training condition F-NT subjects were significantly lower than HC subjects and only in dorsal CA1 (p=0.004) while N-NT subjects' were just under the threshold for significant reduction (p=0.051). Arc protein expression was not enhanced relative to HC subjects in either hippocampal subfield following novel or familiar context exposure and was actually reduced in dorsal CA1.

A separate two-way repeated measure ANOVA was conducted on ventral hippocampus immunohistochemical data with training condition (F-NT, N-NT, HC) `as the between subjects factor and hippocampal subfield (CA1, CA3) as the within subjects factor. Statistical analyses revealed there was a significant main effect of training condition ($F_{(2,43)}$ = 9.393, p=0.001), a significant main effect of subfield, ($F_{(1,43)}$ =41.812, p<0.001), with a significant interaction between condition and subfield, ($F_{(2,43)}$ = 10.114, p=0.001). SNK *post hoc* analysis identified N-NT subjects' mean Arc expression to be significantly different than F-NT (p<0.001) and HC subjects (p<0.001) within CA1 but not CA3. Importantly, F-NT and HC subjects were not significantly different (p=0.688) across either CA1 (p = 0.878) or CA3 (p=0.618) (Figure E2-5b). Arc protein was preferentially enhanced in ventral CA1 following exposure to a novel context (N-NT) and importantly subjects exposed to a familiar context (F-NT) were statistically undifferentiated from HC subjects across the ventral hippocampus.

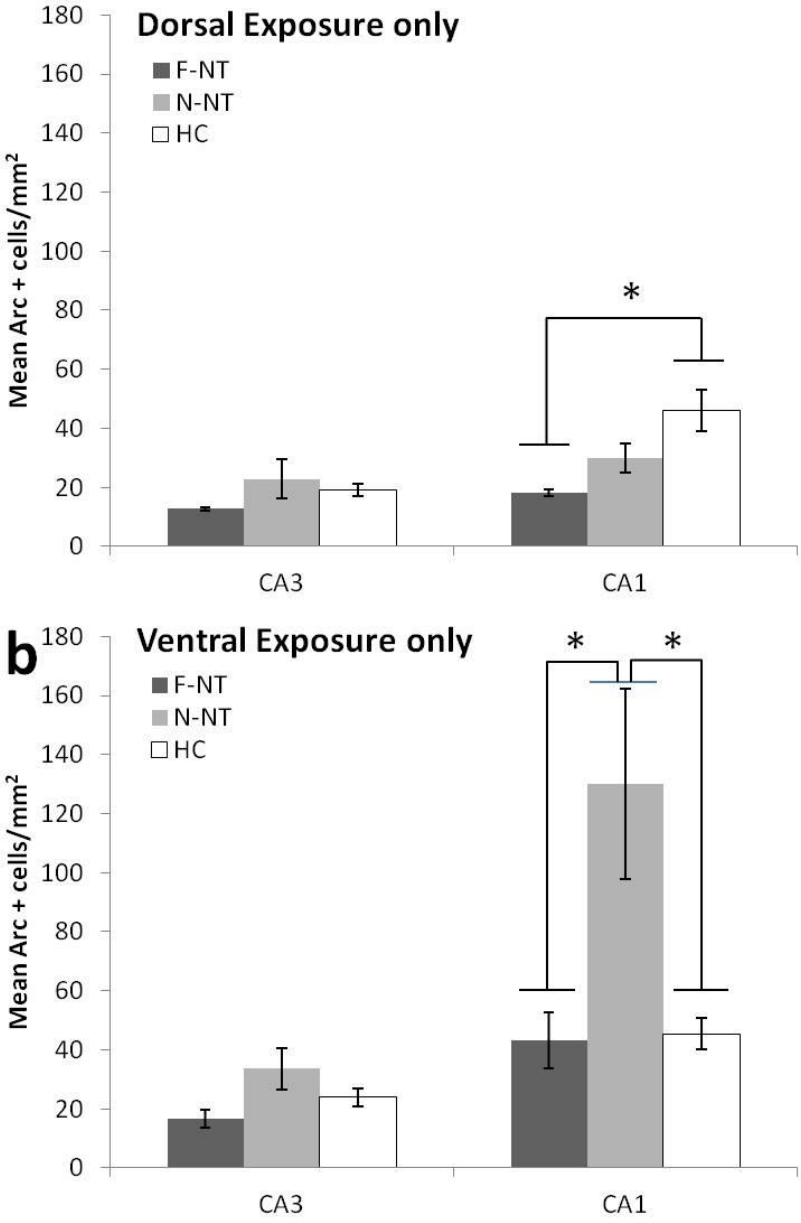

Figure E2-5. Arc positive cells counts in CA3 and CA1 subfields in dorsal and ventral hippocampus. (a) Mean ± SEM Arc positive cells per mm^2 in the dorsal hippocampus. SNK *post hoc* analysis identified HC subjects to be significantly different than F-NT ($p=0.004$) and near significant for N-NT ($p=0.051$). (b) Mean ± SEM Arc positive cells per mm^2 in the ventral hippocampus. SNK *post hoc* analysis identified N-NT subjects' mean Arc expression to be significantly different than F-NT ($p<0.001$) and HC subjects ($p<0.001$) within CA1 but not CA3.

Discussion

The primary goal of the present study was to identify a context pre-exposure period that would reduce Arc protein expression due to novel context exposure. Experiment 1 identified enhancement in Arc protein following novel context exposure which could not be dissociated from enhancement due to fear conditioning. Ten days of pre-exposure to the training context was expected to reduce Arc protein enhancement due to novel context exposure. This initial effect of novel context induced Arc enhancement observed in Experiment 1 was replicated (Figure E2-4) and extended in the current experiment by pre-exposing some subjects to the conditioning apparatus (F-NT); pre-exposure was sufficient to reduce both behavioral (mobility) and neurochemical (Arc protein enhancement) indicators of context novelty.

Animals pre-exposed to the training context were significantly less mobile

Both N-NT and F-NT groups were highly mobile during the first five minutes of exposure (Figure E2-1). This high level of mobility was maintained for the N-NT subjects but not for F-NT subjects, whose mobility decreased across minutes to the end of the trail (Figure E2-1). While the subjects in the current experiment were not exposed to fear conditioning, Barot et al., (2009) demonstrated contextual latent inhibition following fear conditioning after a similar pre-exposure procedure. The latent inhibition effect is thought to reflect a shift in associability away from the pre-exposed relative to a more novel stimulus. Though the specific behavioral and neurobiological mechanisms underlying

latent inhibition are still under investigation (Escobar et al., 2002; Shohamy et al. 2000), the current experiment was not designed to investigate such mechanisms but rather to take advantage of the behavioral effects of familiarizing subjects with contextual stimuli present during subsequent conditioning.

Arc protein expression patterns replicated across Experiment 1 and Experiment 2 for subjects exposed to a novel context

Subjects from both Experiment 1 and Experiment 2 demonstrated similar patterns of Arc protein expression in both the dorsal and ventral hippocampus, most notably the significant enhancement of Arc protein in ventral CA1 relative to HC subjects following novel context exposure. While the handling procedure was modified for Experiment 2 (see methods), both groups of animals were sacrificed after exposure to a novel environment. Interestingly, while Arc protein enhancement due to novel context exposure has been documented in the dorsal hippocampus (Ramirez-Amaya et al., 2005) and has been implicated in place cell stabilization (Guzowski et al., 1999), significant increases in Arc protein relative to HC subjects was not observed in the dorsal hippocampus following novel context exposure in either Experiment 1 or 2. There was significant enhancement in ventral CA1, and some place cells have been identified in this region (Jung et al., 1994), however the role of Arc protein expression in the ventral hippocampus is largely unknown.

Arc protein expression in the dorsal hippocampus is reduced for subjects exposed to a familiar versus novel context

Dorsal hippocampal Arc protein expression was not significantly enhanced relative to HC subjects and within dorsal CA1 Arc protein expression was significantly lower than HC for F-NT subjects and approached significance for N-NT subjects. Others have reported significant increases in Arc mRNA and protein following exposure to a novel environment (Ramirez-Amaya et al., 2005; Vazdarjanova et al., 2006) in both dorsal CA1 and CA3 compared to HC subjects. We did not observe a significant increase in Arc protein following novel or familiar context exposure within either subfield. Inconsistencies in these results across laboratories may be due to a variety of factors. First, there were differences in the context exposure procedures. In our experiment the novel context was an operant conditioning chamber no larger than the animal's home cage, whereas in other studies the novel context was a larger open field enclosure (Ramirez-Amaya et al. 2005; Vazdarjanova & Guzowski 2004; Guzowski et al., 1999); thus the subjects in the present study were investigating a much smaller and dimensionally more similar space to the animals' home cage which may account for the lack of Arc enhancement. Additionally, there were differences in the novel context exposure procedures, particularly with respect to the role of the experimenter in facilitating context exploration. While in the present study subjects were free to explore the context undisturbed, subjects in studies from other laboratories were moved manually around the novel context (Ramirez-Amaya et al., 2005; Guzowski et al., 1999). Yet another procedural difference was the use of Western blot analysis and/or florescent antibodies with optical density measures to determine differences in Arc protein expression

(Lonergan et al., 2010; Ramirez-Amaya et al., 2005; Vazdarjanova et al., 2006), while we utilized colorimetric DAB stains and hand-counted individually stained cells. Given these differences it is difficult to directly compare results across studies. While others have demonstrated Arc protein enhancement in the dorsal hippocampal CA3 and CA1 subfields following novel context exposure, we did not observe this enhancement.

For subjects exposed to a familiar context (F-NT), Arc protein expression was reduced in both CA3 and CA1 compared to HC subjects and this difference was significant in dorsal CA1. While we initially predicted no difference between HC and F-NT Arc protein expression the decrease is slight and ultimately irrelevant for our purposes. Despite F-NT Arc expression being lower than HC values it is also consistently lower than N-NT subjects in CA1 and CA3, though not significantly. Within the dorsal hippocampus, 10 days of context pre-exposure functions to provide a new baseline to dissociate Arc enhancement specific to trace fear conditioning from that induced by novel context exposure.

Arc protein expression in the ventral hippocampus is significantly enhanced for subjects exposed to a novel context in CA1 but not CA3

Significant enhancement in Arc protein expression in the ventral hippocampal CA1 subfield was observed for subjects exposed to a novel context, replicating the “approaching significance” effect identified in Experiment 1. However the current experiment identified significant enhancement only in ventral CA1 suggesting a greater role for Arc protein in this region relative to

ventral CA3 in novel context exposure and exploration. While the role of Arc protein expression in the ventral hippocampus is largely unknown, as outlined in experiment 1, neophobia induced by novel context exposure suggests a potential role for amygdala modification of hippocampal Arc expression during exploration of a potentially aversive and unknown environment. Preferential expression in ventral CA1 could be due to the dense, reciprocal connections to the amygdala specific to that region while ventral CA3 primarily receives input. Arc protein enhancement may be greater in CA1 due that reciprocal connectivity between that region and the amygdala. Meanwhile, F-NT subjects demonstrated levels of Arc protein expression nearly identical to that of HC subjects in both ventral CA1 and CA3 and Dorsal CA3. Dorsal CA1 Arc protein following familiar context exposure was significantly lower than HC subjects but as discussed above this difference will not preclude us from using this same pre-exposure period in future experiments in order to dissociate Arc enhancement due to novel context exposure from that induced by trace fear conditioning. The pre-exposure procedure used in this experiment was sufficient in preventing novel context induced Arc protein enhancement in ventral CA1, and with the exception of dorsal CA1, F-NT subjects demonstrated Arc protein expression patterns nearly identical to that of HC subject.

Conclusion

The pre-exposure procedure from the current experiment reduced exploratory behavior and Arc protein enhancement in ventral CA1 induced by exposure to the novel training chamber. Animals that were pre-exposed to the

conditioning context (F-NT) demonstrated levels of Arc protein statistically undifferentiated from that of HC subjects in all examined subfields except dorsal CA1, and that was a reduction. N-NT subjects demonstrated a similar near-significant reduction in dorsal CA1 relative to HC controls but also demonstrated a significant enhanced in ventral CA1, which was prevented by the pre-exposure procedure for F-NT subjects. Hence this pre-exposure procedure can now be utilized prior to fear conditioning allowing us to identify Arc protein enhancement specific to the fear conditioning task and independent of context exposure, as the familiar context does not induce Arc protein expression in its own right. Additionally pre-exposure should produce robust contextual latent inhibition biasing the animal away from learning about the context-US association, promoting greater CS-US learning, and ideally enhancing hippocampal Arc protein expression specific to the explicit CS-US contingency. The current experiment replicated and extended observations of Arc protein enhancement due to novel context exposure. Arc protein expression was significantly enhanced in ventral CA1 following novel context exposure. These data are consistent with a potential role for ventral hippocampal Arc protein expression and synaptic plasticity in novel environment exploration.

Comparing specific patterns of Arc protein induced by conditioning after our pre-exposure period will provide a new and unique method to address the primary goal of isolating which aspect of the trace fear conditioning experience induces learning dependent Arc protein expression (CS-US, context-US, context only representations) across subfields; and provide an important contribution for

understanding the specific role of the hippocampus in trace fear conditioning as well as new predictions about the conditions under which a learning experience recruits the hippocampus.

Experiment 3. Arc protein expression following trace fear conditioning in a novel or familiar context

In Experiment 1, we were unable to dissociate Arc protein enhancements due to trace fear conditioning from that induced by exposure to a novel context. With this in mind, the results of Experiment 2 suggest that 10 days of pre-exposure to a context was sufficient to reduce novel context-induced mobility and Arc protein expression in ventral CA1, suggesting that context familiarity can modulate Arc expression within discrete subfields of the hippocampus. Based on the outcomes of Experiment 2 we are now better able to address the initial question proposed in Experiment 1, which sought to identify the specific subfields within the hippocampus that show an enhancement in Arc protein expression following trace fear conditioning. First, pre-exposure importantly removed the influence of novel context-induced Arc expression in ventral hippocampus as described above. Second, the procedure is also expected to produce contextual latent inhibition in fear conditioning (Barot et al., 2009). By shifting US associability away from the context (context-US) (Escobar et al., 2002), learning about the explicit auditory CS should be maximized (CS-US). As such Arc expression within hippocampal subfields preferentially activated by the CS may also be maximized for subjects trained in a familiar context compared to one which is novel.

In the current experiment, subjects were trained in trace fear conditioning (described in general methods) in either a novel or familiar context. Briefly, we expect that trace fear conditioning is likely supported by CA3 of the ventral

hippocampus and reciprocal connections between ventral CA1and the amygdala, as well as reciprocal ventral CA1 connections to dorsal CA3. After controlling for novel context exposure, we expect to see enhancements in Arc protein expression across these subfields for subjects trained in trace fear conditioning. Contextual latent inhibition is expected for subjects trained in a familiar context compared to one which is novel. However, since subjects are pre-exposed to the context and not the explicit auditory CS, the observed effects of contextual latent inhibition may not be directly apparent until the testing sessions.

This indeed this was observed. Subjects trained in TFC in a familiar context demonstrated a significant reduction in contextually elicited fear compared to subjects trained in a novel context. However, these data will be addressed in Chapter 4 in a cross experiment analysis of Arc protein expression as well as CS and context elicited fear. While contextually elicited fear will not be reported in this section, contextual pre-exposure is expected to be reflected in a difference in freezing to the CS during tone testing. If subjects are pre-exposed to the conditioning context then acquisition of the context-US association should be inhibited and facilitate acquisition of the more novel CS-US association (Boughner et al., 2004) in so that during tone testing the CS elicits more freezing for subject trained in a familiar context compared to one which is novel.

Methods

Variations in procedures to general methods are outlined below.

Subjects. 23 male Sprague-Dawley rats (Harlan, Indianapolis, IN), weighing 250-300g, served as subjects.

Procedure

Behavioral procedures. All trained subjects (n=20) were treated identically to rats from Experiment 2 prior to trace fear conditioning (outlined in the Context Pre-exposure section above). After the context pre-exposure procedure these animals were then trained in auditory trace fear conditioning (TFC) in a pre-exposed, familiar (**F-TFC**, n=10) context, or one which was novel (**N-TFC**, n =10). Auditory trace fear conditioning was carried out using the same parameters as Experiment 1 and is outlined in the general methods section. Following training a subset of the subject from both groups (F-TFC: n=5; N-TFC: n=5) were returned to their home cage for one hour prior to sacrifice and tissue processing for immunohistochemical analysis of Arc protein expression. Three home cage (**HC**) subjects were also sacrificed. The remaining subjects (F-TFC: n=5; N-TFC: n=5) were not sacrificed for immunohistochemical analyses on that day. Instead these animals were tested 24hr later in a novel context for fear conditioned to the tone CS, and again 24hr later (48 hours after training) for contextually elicited fear. One hour after this final behavioral test, these remaining subjects were sacrificed for tissue processing and immunohistochemical analysis of Arc protein expression. These data will be discussed in an independent section.

Immunohistochemistry and Histology were carried out according to the General Methods section.

Results

Behavioral training and testing

As described previously, two groups of subjects (F-TFC, N-TFC) trained in auditory trace fear conditioning were observed for a freezing CR. After training, one F-TFC subject was removed due to health reasons, leaving final sample sizes for analysis of training data (F-TFC: n=9; N-TFC: n=10). Conditioned responding for the remaining subjects (F-TFC: n=5; N-TFC: n=5) was also measured during testing sessions examining fear conditioned to the tone CS and context testing. The mean (±SEM) percentage of freezing exhibited by F-TFC and N-TFC during training and tone testing is presented in Figure E3-1.

Training. Two separate two-way repeated measures ANOVAs were conducted with training context (N-TFC, F-TFC) as the between subjects factor and trial period (ITI, CS, Trace) as the within subjects factor. For Trial 1 (Figure E3-1a) statistical analyses revealed there was no main effect of training context ($F_{(1,56)}$ = 1.118, p=0.305), no significant main effect of trial period ($F_{(2,56)}$ = 1.118, p=0.339), with no significant interaction between training context and trial period ($F_{(2,56)}$ = 1.118, p=0.339). There was little to no freezing for either N-TFC or F-TFC subjects during the first trial prior to the first US delivery.

For Trials 2-7 (Figure E3-1b) statistical analyses revealed there was no main effect of training context ($F_{(1,56)}$ = 0.132, p=0.720), a significant main effect of trial period ($F_{(2,56)}$ = 32.700, p<0.001), and no significant interaction between training context and trial period ($F_{(2,56)}$ = 0.176, p=0.840). SNK *post hoc* analysis

identified significantly higher levels of freezing during the trace interval compared to during the CS ($p<0.001$) and ITI ($p<0.001$) however CS and ITI periods were not significantly different ($p=0.270$). While there were no differences between N-TFC and F-TFC subjects during training in trace fear conditioning both groups froze significantly more during the trace interval than any other trial period.

Tone testing. The data from ITI-1 during tone testing are interpreted as a "baseline" level of freezing to the novel testing context and are separated from the other ITI freezing data (Figure E3-1c). In order to determine if context novelty significantly effects freezing to the tone CS during testing, another two-way repeated measures ANOVA was conducted with training context (N-TFC, F-TFC) as the between subjects factor and trial period (ITI-1 vs. ITI, CS, Trace) as the within subjects factor. Statistical analyses revealed there was no significant main effect of training context ($F_{(1,39)} = 0.201$, $p=0.669$), a significant main effect of trial period ($F_{(3,39)} = 57.276$, $p<0.001$), with no significant interaction between training context and trial period ($F_{(3,39)} = 0.634$, $p=0.600$). SNK *post hoc* analysis identified significant differences between all trial periods ($p <0.001$; CS v. ITI-1 $p=0.009$) except during trace and ITI periods ($p=0.280$). While there was no difference between N-TFC and F-TFC subjects during testing to a tone CS both subjects froze significantly less during the CS period. While not significantly different there is a trend toward a further reduction in freezing to the CS for subjects trained in a familiar context (F-TFC).

Context testing. Context testing behavioral data was collected and subjects trained in familiar context demonstrated robust contextual latent inhibition,

however these behavioral data will be reported in the cross experiment analysis in Chapter 4.

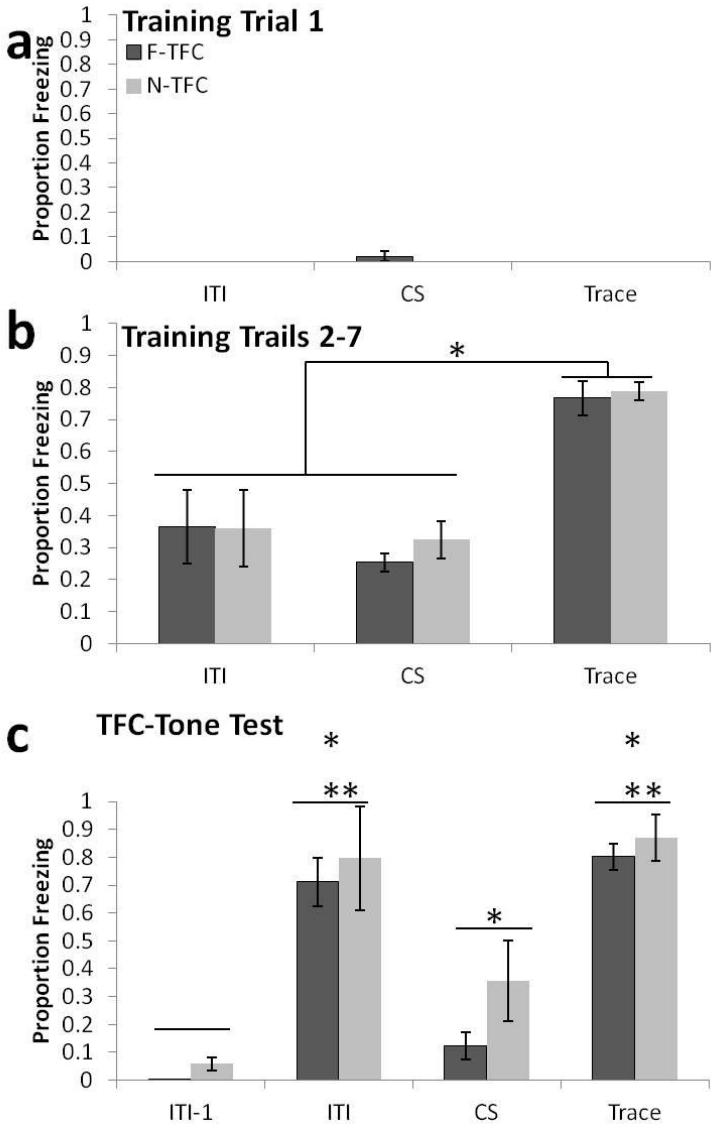

Figure E3-1: Proportion of session freezing for F-TFC and N-TFC subjects across different trial periods. (a) Trial one of training in TFC (b) Trials 2-7 of training in TFC with significantly more freezing during the trace interval compared to during the CS ($p<0.001$) and ITI ($p<0.001$). (c) Freezing to the tone CS 24 hours after training with significant differences between all trial periods ($p <0.001$ (ITI-solid line) (trace-dashed line); CS v. ITI-1 $p=0.009$ (dot-dash line)) except during trace and ITI periods ($p=0.280$).

Immunohistochemical examination of regional patterns of Arc expression

Hippocampal neuronal cells stained positive for Arc protein were quantified across the septotemporal and transverse axes of both dorsal and ventral hippocampus. Statistical analyses are reported for Arc positive cell counts/mm^2.

Typically Arc positive cell counts are averaged across 3 independent counters blind to the experimental conditions of the subjects. For one counter their threshold for identifying a cell as positively stained was much lower than the other two counters resulting in much high numbers of cells counted, particularly in dorsal CA1. Out of the complete range of values observed in dorsal CA1 for 12 different HC subjects the number of Arc positive cells identified by this counter were determined to be outliers on two out of three occasions by Dixon's outlier test: one HC subject ($Z_{(31)} = 2.923$, $p<0.05$) second HC subject ($Z_{(30)} = 2.908$, $p<0.05$). As a result of this all of this individual's counts were excluded and the data presented here is averaged across two independent counters instead of three.

<u>Arc positive cells per mm^2 in dorsal and ventral hippocampal subfields CA1 & CA3 compared to home cage control subjects</u>

In order to determine if context pre-exposure significantly enhances Arc protein expression compared to HC control subjects in dorsal hippocampus, a two-way repeated measure ANOVA was conducted on immunohistochemical data (Figure E3-2a) with training context (F-TFC, N-TFC, HC) as the between

subjects factor and hippocampal subfield (CA1, CA3) as the within subjects factor. Statistical analyses revealed there was no main effect of training context ($F_{(2,41)}$ = 1.757, p=0.201), no significant main effect of subfield, ($F_{(1,41)}$ = 2.458, p=0.134), but a significant interaction between training context and subfield, ($F_{(2,41)}$ = 16.761, p<0.001). SNK *post hoc* analysis identified Arc protein to be significantly enhanced for F-TFC and N-TFC subjects relative to HC controls but only in dorsal CA3 (p=0.005, p<0.001, respectively). Arc protein was enhanced in subjects trained in TFC relative to HC subjects in dorsal CA3 yet there were no significant differences in protein expression due to training in familiar context.

A separate two-way repeated measure ANOVA was conducted on ventral hippocampus immunohistochemical data (Figure E3-2b) with training context (F-TFC, N-TFC, HC) as the between subjects factor and hippocampal subfield (CA1, CA3) as the within subjects factor. Statistical analyses revealed there was a significant main effect of training context ($F_{(2,41)}$ = 29.876, p<0.001), a significant main effect of subfield, ($F_{(1,41)}$ = 105.099, p<0.001), and a significant interaction between training context and subfield, ($F_{(2,41)}$ = 17.611, p<0.001). SNK *post hoc* analysis identified significant enhancements in Arc protein relative to HC subjects for F-TFC and N-TFC subjects in CA1 (p<0.001) but also now in CA3 (p=0.029, p=0.015, respectively). Arc protein was enhanced in subjects trained in TFC relative to HC subjects in both dorsal CA3 and CA1, however there were again no significant differences in Arc protein expression due to training in a familiar context.

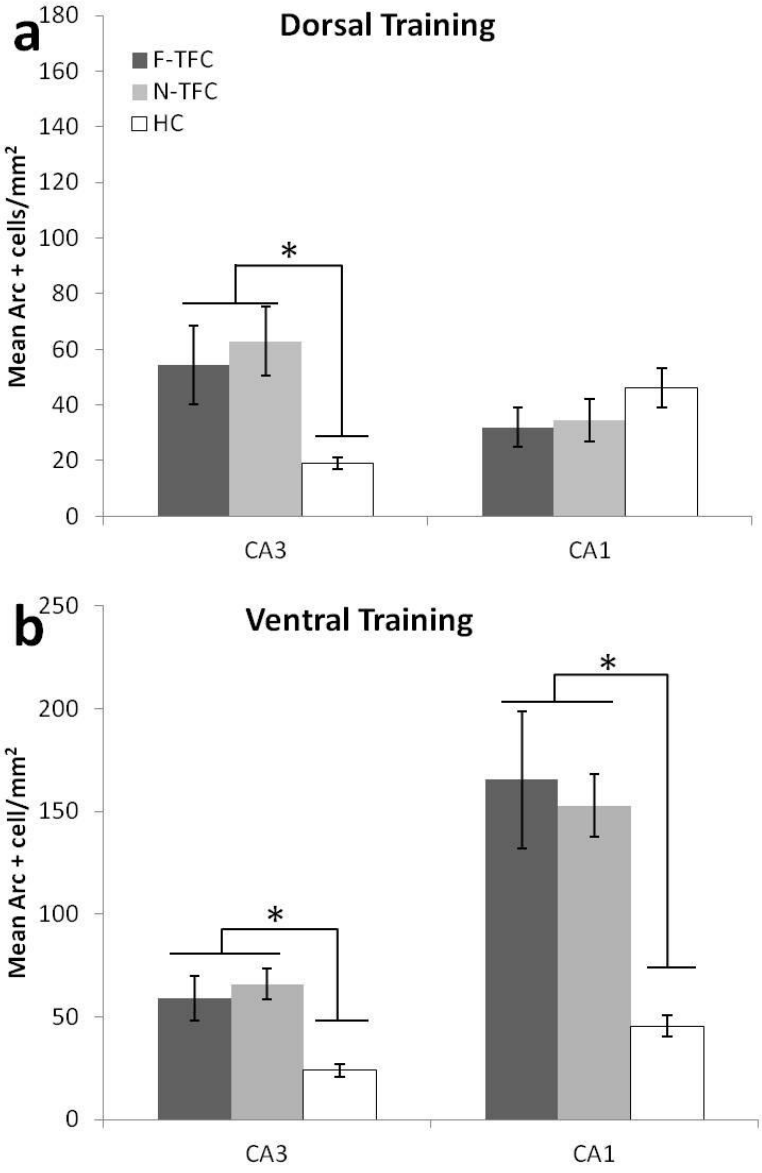

Figure E3-2: Arc positive cells counts per mm2 in CA3 and CA1 subfields in dorsal and ventral hippocampus. (a) Mean ± SEM Arc positive cells per mm2 in the dorsal hippocampus was significantly enhanced in F-TFC and N-TFC subjects relative to HC controls in dorsal CA3 ($p=0.005$, $p<0.001$, respectively). (b) Mean ± SEM Arc positive cells per mm2 in the ventral hippocampus was significantly enhanced relative to HC subjects for F-TFC and N-TFC subjects in both CA1 ($p<0.001$) and CA3 ($p=0.029$, $p=0.015$, respectively).

Discussion

The current experiment sought to identify which subfields of the dorsal and ventral hippocampus show an enhancement in Arc protein for animals trained in trace fear conditioning independent from Arc protein enhancement due to exposure to a novel context. As stated above we expected enhancements in Arc protein following trace fear conditioning in CA3 of the dorsal hippocampus and both CA1 and CA3 of the ventral hippocampus. Both familiar (F-TFC) and novel (N-TFC) context trained subjects are expected to show enhancements in Arc protein relative to HC controls. Arc is expected to be reduced in F-TFC compared to N-TFC subjects in the same subfields enhanced due to novel context exposure identified in Experiment 2 (ventral CA1, possibly dorsal CA3). Behaviorally we do not expected context exposure to significantly affect conditioned responding during training. However, as discussed above, we do expect to shift learning toward the explicit CS-US associations compared to context-US associations and observe more conditioned responding to the CS for F-TFC subjects during tone testing.

Behavioral measures of freezing did not differ between F-TFC and N-TFC subjects during training or testing

Both groups were trained in trace fear conditioning and as expected there was not a significant difference in freezing due to context familiarity during training. This was expected since only the context was pre-exposed, not the explicit CS, and as such latent inhibition would likely not be observed during CS training but during the subsequent CS alone and context alone conditioned response testing. During all trial periods both F-TFC and N-TFC subjects showed

substantial conditioned responding with significantly more freezing during the trace interval. This pattern of relatively more freezing during the trace interval relative to other periods was also observed during testing. This is a common observation in our laboratory (Czerniawski et al. 2011, 2012), and is consistent with the notion that the trace interval may serve a different kind of predicative function for the animal in trace conditioning. Overall, a lack of a difference in freezing behavior between N-TFC and F-TFC subjects during training supports our initial hypothesis.

Due to context pre-exposure we expected a shift in associability toward the CS during tone testing for subjects trained in a familiar context (Broughner et al., 2004). More specifically, F-TFC subjects were expected to show more freezing during CS testing than N-TFC subjects. For F-TFC subjects the associative strength of the context-US contingency was expected to be minimized due to 10 days of context pre-exposure prior to trace fear conditioning, producing a shift in associability toward the explicit CS-US contingency compared to the context-US contingency. The data did not support this hypothesis as both F-TFC and N-TFC freezing was not significantly different across trial periods. However, for both groups freezing was significantly reduced during the CS period compared to any other trial period. This again supports the interpretation of an alternative function for the CS in trace conditioning. In a complete opposite prediction from our hypothesis, F-TFC subjects CS freezing was even further reduced than N-TFC subject, though not significantly different. As discussed in Experiment 1 there is a possibility that the CS may serve as a

kind of “safety signal” during trace conditioning so that the CS comes to predict the absence of the US and that learning this differential prediction is enhanced when conditioning occurs in a familiar context. In this interpretation, the data does support enhanced CS learning for subjects trained in a familiar context however it is not an enhancement of CS learning as an excitatory stimulus predicting the US, but a conditioned inhibitor predicating the explicit absence of the US. While freezing to the CS was not significantly lower for F-TFC subjects there was trend. This will be readdressed and discussed in more detail in Chapter 4.

Arc protein expression

Subjects trained in trace fear conditioning demonstrated significant enhancements in Arc protein in CA3 of the dorsal hippocampus and both CA1 and CA3 of the ventral hippocampus relative to HC control subjects supporting our initial hypothesis. However there were no significant reductions in F-TFC Arc protein expression compared to N-TFC subject who were expected to show elevated Arc protein in ventral CA1 reflecting enhancement elicited by both the novel context and trace fear conditioning.

Dorsal hippocampus Arc protein expression

Enhancement in Arc positive cells counts in CA3 but not CA1 is generally consistent with our initial hypothesis. Evidence outlined in Experiment 1 and replicated here in Experiment 3 shows significant Arc protein enhancement in dorsal CA3 and not CA1. This was expected for both F-TFC and N-TFC groups

as both were trained in trace fear conditioning. As in Experiment 1, this suggests that Arc expression within dorsal CA3 may reflect contextual components of trace fear conditioning (context-US associations) as subjects trained in fear conditioning also exhibit contextually elicit fear (not reported here). Unexpected and inconsistent with our hypothesis was the lack of reduction in any subregion for F-TFC subjects compared to N-TFC subjects. A reduction in dorsal CA3 in F-TFC relative to N-TFC subjects was a prediction based on the data from Experiment 2 showing a minor, and not statistically significant, elevation in dorsal CA3 for subject exposed to a novel context. Hence we expected that when trace fear conditioning occurred in addition to exposure to the novel context that we would observe an additive effect on Arc protein enhancement. Arc protein enhancement for N-TFC subjects was expected to reflect both novel context and trace fear conditioning induced Arc protein while Arc enhancement for F-TFC subjects could only be due to trace fear conditioning. The lack of a difference between these two groups suggests that Arc expression is not additive in the way in we hypothesized, however given that Arc protein was not significantly enhanced in dorsal CA3 relative to HC subjects in Experiment 2 then Arc protein expression may still be additive in other subfields where there was a significant enhancement (ventral CA1). Arc protein was significantly enhanced in dorsal CA3 for trained subjects supporting our initial hypothesis however Arc expression within CA3 was not additive with no meaningful differences in Arc protein expression between familiar and novel context trained subjects.

Ventral hippocampus Arc protein expression

Enhancement in Arc positive cells counts in CA3 and CA1 is generally consistent with our initial hypothesis. Arc protein expression in the ventral hippocampus was elevated in both CA3 and CA1 compared to HC controls for subjects trained in trace fear conditioning supporting our initial hypothesis. This is consistent with both the results of Experiment 1 and anatomical data identifying dense reciprocal connections between CA1 and the amygdala. Additionally ventral CA1 also provides the primary pathway by which amygdala inputs into the ventral hippocampus reach the dorsal hippocampus which is required to support CFC (Corcoran and Maren, 2001) and in some cases TFC (Czerniawski et al., 2011).

Similar to Arc expression in the dorsal hippocampus, there was an unexpected lack of a reduction in F-TFC subjects compared to N-TFC subjects within subfields significantly enhanced by novel context exposure. Here we expected a greater enhancement in ventral CA1 specifically for N-TFC subjects as this subfield showed significant enhancement in Arc protein following novel context exposure. Training subjects in trace fear conditioning after exposing them to a novel context did not have an additive effect on Arc protein enhancement in CA1 or CA3. However Arc protein was significantly enhanced in CA3 relative to HC subjects for subjects trained in fear conditioning while this was not the case for subjects exposed to a novel context (Experiment 2). This suggests that Arc protein expression in ventral CA3, and not ventral CA1, may be unique to TFC. Given the potential role of dorsal CA3 and not dorsal CA1 supporting contextual components of TFC, these data suggest that ventral CA3, and not ventral CA1, may support CS components of TFC. Here the neuronal ensemble representing

the CS component of CS-US association underlying TFC may extend to the ventral hippocampus CA3 during trace conditioning. This suggests that neurons within ventral CA3 and not CA1 may have distinct receptive fields and tuning curves for the trained tone CS and not other tones. If so, then ventral CA1 Arc expression may not be specific to TFC and, while still a critical component for potential fear based learning, (neophobia and ventral CA1 – see Experiment 1 discussion) ventral CA1 Arc protein enhancement may not uniquely represent any of the various learned associations during TFC (CS-US or context-US). Instead CA1 may be the primary relay station by which amygdala activity reaches dorsal CA3 to support contextual learning (context-US) and ventral CA3 to support explicit CS learning (CS-US).

Ultimately these notions are speculative as we do not know if this pattern of Arc protein enhancement is specific to TFC. A similar pattern of Arc protein may also occur following delay or contextual fear conditioning in a familiar context. If subjects trained in CFC show a similar pattern of activity in ventral CA3 then it is unlikely that activity in that region is unique to explicit-CS learning as no explicit CS is presented during contextual fear conditioning. Additionally if a similar pattern of Arc protein is observed after DFC in the ventral hippocampus then it is unlikely that ventral CA3 Arc expression reflects CS-US processing since preventing Arc expression in the ventral hippocampus has no effect on DFC (Czerniawski et al., 2011). While it appears that TFC, after controlling for novel context exposure, preferentially enhances Arc protein in dorsal CA3 and ventral CA1 and CA3, without comparing these results to other paradigmatically

similar fear conditioning tasks, it is unclear if Arc protein enhancement within these regions is unique to TFC.

Conclusion

Subjects trained in TFC in either a novel or familiar context demonstrated similar levels of conditioned responding during both training and tone testing. During tone testing freezing to the CS was reduced compared to both the trace and ITI periods and this reduction was even greater in subjects trained in a familiar context, suggesting a potential alternative functional role for the CS in trace conditioning.

Arc protein expression was enhanced in regions hypothesized to support TFC (dorsal CA3, ventral CA1 and CA3). However, Arc protein expression induced by TFC is not additive to Arc protein induced by novel context exposure as expression was not different for subjects trained in novel or familiar context across any subfield. However, both dorsal and ventral CA3 Arc expression seems unique to subjects trained in TFC as these subfields do not show significant enhancement in Arc protein following novel context exposure; yet it is presently unclear if similar patterns of Arc protein would occur following a hippocampal independent task with an explicit CS (delay conditioning) or another hippocampal dependent task without an explicit CS (contextual conditioning). These issues are addressed in Experiment 4.

Experiment 4. Arc protein expression following contextual and delay fear conditioning in a novel or familiar context

Analyses from Experiments 2 and 3 suggest that subjects exposed to a novel context show significant enhancement of Arc protein in ventral CA1, and after controlling for novel context exposure, TFC enhances hippocampal Arc protein expression in dorsal and ventral CA3 and ventral CA1 relative to HC control subjects. Based on evidence reviewed above, this suggests that Arc protein expression in dorsal CA3 may reflect the contextual components (context-US) of TFC while ventral CA3 Arc expression reflects explicit CS components (CS-US). However, it is unclear if the change in Arc protein in either of these subfields is specific to the acquisition of trace fear conditioning *per se* without comparing these results to paradigmatically similar conditioning tasks, for example contextual and delay fear conditioning.

CFC and DFC tasks were selected as comparisons to TFC as they allow for a direct examination of whether Arc protein expression within a given subfield (dorsal CA3, ventral CA3) is unique to a particular component (context-US, CS-US) of the trace fear conditioning task. For example, if a similar pattern of Arc protein expression is observed after hippocampal dependent CFC as in TFC then Arc protein expression in ventral CA3, induced by TFC, is likely not reflecting explicit tone CS processing as there is no explicit tone CS presented in CFC. Additionally, if similar patterns of Arc protein expression are observed after DFC, then Arc protein in ventral CA3 is also likely not reflecting explicit tone CS processing as acquisition of the CS-US contingency in DFC is hippocampal

independent. It is possible that DFC and CFC will both produce enhancements in Arc protein in dorsal CA3 as both of these tasks produce contextual conditioning and contextually-elicited fear. However enhancement in dorsal CA3 may still differ based on the strength of contextual conditioning as evident through differences in contextually-elicited fear between TFC, CFC and DFC. While individual expression patterns will be analyzed in the context of the current experiment, a direct comparison of Arc protein enhancements between subjects trained in TFC, CFC, and DFC will be included in the next section of the manuscript.

Results from Experiment 3 also suggest that patterns of Arc protein expression for subjects trained in TFC were not significantly affected by context familiarity. However, it is unclear if the context pre-exposure procedure used in Experiment 3 will have a similar effect (or lack or effect) on conditioned responding and Arc protein expression for DFC and CFC subjects. Specifically, Arc protein expression and freezing during CFC, and not DFC, may be particularly sensitive to context pre-exposure for a variety or reasons. First, during CFC, the context itself is the primary predictor of the US, and it is now well established that this context-US association is dependent on the hippocampus (Fanselow and Poulos, 2005, Maren, 2001, reviewed in Orsini & Maren, 2012). Furthermore, by selectively inhibiting and activating dorsal hippocampal neurons, CFC can be directly inhibited (Tanaka et al., 2014) or facilitated to produce artificial contextual memory (Ramirez et al., 2013), all which highlight the critically important role of the hippocampus in CFC.

Second, emerging evidence suggests that while contextual representations are initially dependent on dorsal hippocampus, they are ultimately consolidated in other regions, eventually becoming hippocampal independent (Anagnostaras et al., 1999; Kim and Fanselow, 1992; Frankland et al., 2006; but see Sutherland and Lehmann, 2011; Sutherland et al., 2010). Hence, our pre-exposure period may allow for the consolidation of the contextual representation prior to contextual fear conditioning, which could in turn significantly impact both Arc protein expression as well as conditioned responding. This notion is supported by behavioral data demonstrating the well-established role of context pre-exposure in ameliorating the immediate shock deficit (Fanselow, 1986; Fanselow, 1990). Hence it is likely that context pre-exposure will have a more significant effect on the acquisition of CFC compared to TFC given that in CFC the context is directly paired with the US.

While context pre-exposure prior to CFC is likely to have a very different impact on conditioned responding and Arc protein expression than it did on subjects trained in TFC, differences between TFC and DFC with respect to the effects of context pre-exposure are less clear. The assumption that context pre-exposure allows from consolidation of contextual representations prior to conditioning, as suggest above, would likely not have an impact on freezing and Arc protein expression for subjects trained in DFC. The formation of contextual representations, as previously described, is largely a hippocampal dependent process and given that acquisition of DFC is largely considered to be hippocampal independent (Corcoran & Maren 2001), context pre-exposure is not

expected to significantly affect behavior or Arc protein for subjects trained in DFC.

In order to determine whether the patterns of Arc protein expression in dorsal and ventral CA3 following TFC are unique to context-US or explicit CS-US components of conditioning, and to determine whether context pre-exposure has a similar effect on either behavior or Arc protein enhancement as it did in Experiment 3, subjects were trained in either CFC or DFC in a pre-exposed (familiar) or novel context. We expect context pre-exposure to significantly affect Arc protein expression across both dorsal and ventral hippocampal subfields, as well as significantly affect freezing during fear conditioning for subjects trained in CFC but not DFC. Subjects trained in CFC and DFC in a familiar context in the current experiment, like those trained in TFC, demonstrated robust contextual latent inhibition, However these data will be addressed in Chapter 4 in a cross experiment analysis of Arc protein expression and a behavioral analysis of CS and context elicited fear.

Methods

Variations in procedures to general methods are outlined below.

Subjects. 59 male Sprague-Dawley rats (Harlan, Indianapolis, IN), weighing 250-300g, served as subjects.

Procedure

Behavioral procedures. Subjects were trained in either contextual fear conditioning (CFC) (n=30), delay fear conditioning (DFC) (n=20) or served as home cage controls (HC) (n=9). Fear conditioning occurred following pre-exposure procedures identical to those used in Experiment 2 and 3. Thus the groups comprising the present experiment included animals trained in CFC or DFC in a pre-exposed, familiar context (F-CFC: n=15; F-DFC: n=10), or novel context (N-CFC: n=15; N-DFC: n=10). Training and testing parameters specific to CFC and DFC are outlined in the General Methods. Following training a subset of subjects from both groups (F-CFC: n=5, N-CFC: n=5; F-DFC: n=5, N-DFC: n=5) were returned to their home cage for one hour prior to sacrifice and tissue processing for immunohistochemical analysis of Arc protein expression. The remaining subjects were not sacrificed for immunohistochemical analyses on that day. Instead, these animals were tested 24hr later in a novel context for fear conditioned to the tone CS (F-CFC: n=10, N-CFC: n=10; F-DFC: n=5, N-DFC: n=5). Twenty-four hours later (48 hours after training) a second testing session was conducted back in the original training context to assess contextually elicited fear and contextual latent inhibition, though these data will be reviewed in Chapter 4 (F-CFC: n=5, N-CFC: n=5; F-DFC: n=5, N-DFC: n=5).

Immunohistochemistry and Histology were carried out according to the General Methods section.

Results

Behavioral training and testing

Four groups of subjects (F-CFC, N-CFC, F-DFC, N-DFC) were trained in either contextual or delay fear conditioning in a familiar (pre-exposed) or novel context and observed for a freezing CR. One N-CFC subject and 2 N-DFC subjects were removed due to a technical failure to deliver the footshock US during training. Conditioned responding for some subjects was also measured during a testing session examining fear conditioned to the tone CS. CFC and DFC behavioral data are analyzed separately. The mean (±SEM) percentage of freezing exhibited by F-CFC and N-CFC during training and tone testing is presented in Figure E4-1 and Figure E4-2 for F-DFC and N-DFC subjects. Final sample sizes for statistical analyses are noted in each section. Refer to experiment 1 and General Methods for description and justification of data analysis.

Contextual fear conditioning

Data for subjects trained in CFC was analyzed identically to TFC in that the conditioning session was divided in to trial periods (ITI, CS, Trace). This allows for a direct comparison to TFC trial periods even though during CFC no explicit stimuli are presented. Again, no stimuli are presented during CFC, and there are no actual ITIs, CSs, or trace intervals however for the sake of analysis and data presentation, freezing behavior was divided into these different trial periods. For further justification refer to the General Methods.

Training. Final sample sizes for training were F-CFC: n=15, N-CFC: n=14. Two separate two-way repeated measures ANOVAs were conducted with training

context (N-CFC, F-CFC) as the between subjects factor and trial period (ITI, CS, Trace) as the within subjects factor. For Trial 1 (Figure E4-1a) there was no freezing for either N-CFC or F-CFC subjects during the first trial so no statistical analyses were conducted.

For Trials 2-7 (Figure E4-1b) statistical analyses revealed there was a main effect of training context ($F_{(1,86)} = 7.998$, p=0.009), a significant main effect of trial period ($F_{(2,86)} = 32.636$, p<0.001), and no significant interaction between training context and trial period ($F_{(2,86)} = 1.153$, p=0.323). SNK *post hoc* analysis identified significantly higher levels of freezing for N-CFC subjects compared to F-CFC subjects. Both groups demonstrated significantly higher levels of freezing during both the trace interval and CS period compared to the ITI (p<0.001) however trace and CS periods were not significantly different (p=0.276). N-CFC subjects froze more than F-CFC subjects and for both groups there was more freezing during the CS and trace interval periods than the ITI. Remember that no explicit CS is presented in CFC so the only difference in trial periods is their temporal proximity to the US.

Tone Testing. While no tones were presented during contextual fear conditioning, in order to provide a parallel assessment and testing protocol for all subjects trained in a fear conditioning task (TFC, DFC, CFC), all subjects not sacrificed after training were test for tone elicited freezing. Final sample sizes for tone testing were F-CFC: n=10, N-CFC: n=9. As before the data from ITI-1 during tone testing are interpreted as a “baseline” level of freezing to the novel testing context and are separated from the other ITI freezing data (Figure E4-1c). In

order to determine if context novelty significantly affected freezing to the tone CS during testing, another two-way repeated measures ANOVA was conducted with training context (N-CFC, F-CFC) as the between subjects factor and trial period (ITI-1, ITI, CS, Trace) as the within subjects factor. Statistical analyses revealed there was a significant main effect of training context ($F_{(1,75)}$ = 6.084, p=0.025), a significant main effect of trial period ($F_{(3,75)}$ = 5.062, p=0.004), and a significant interaction between training context and trial period ($F_{(3,75)}$ = 3.759, p=0.016). SNK *post hoc* analysis identified significantly higher levels of freezing for N-CFC subjects compared to F-CFC subjects but only during the ITI (p<0.001). Significant differences between trial periods were only observed for N-CFC subjects with more freezing during ITI compared to ITI-1 (p=0.004) and CS periods (p<0.001) and more freezing during the trace interval than CS period (p=0.020). Despite the lack of a tone CS during conditioning there was still freezing during tone testing and significantly more freezing for subjects trained in contextual fear conditioning in a novel context (N-CFC) however the freezing response did not occur during the presentation of the CS suggesting generalized contextually-elicited fear.

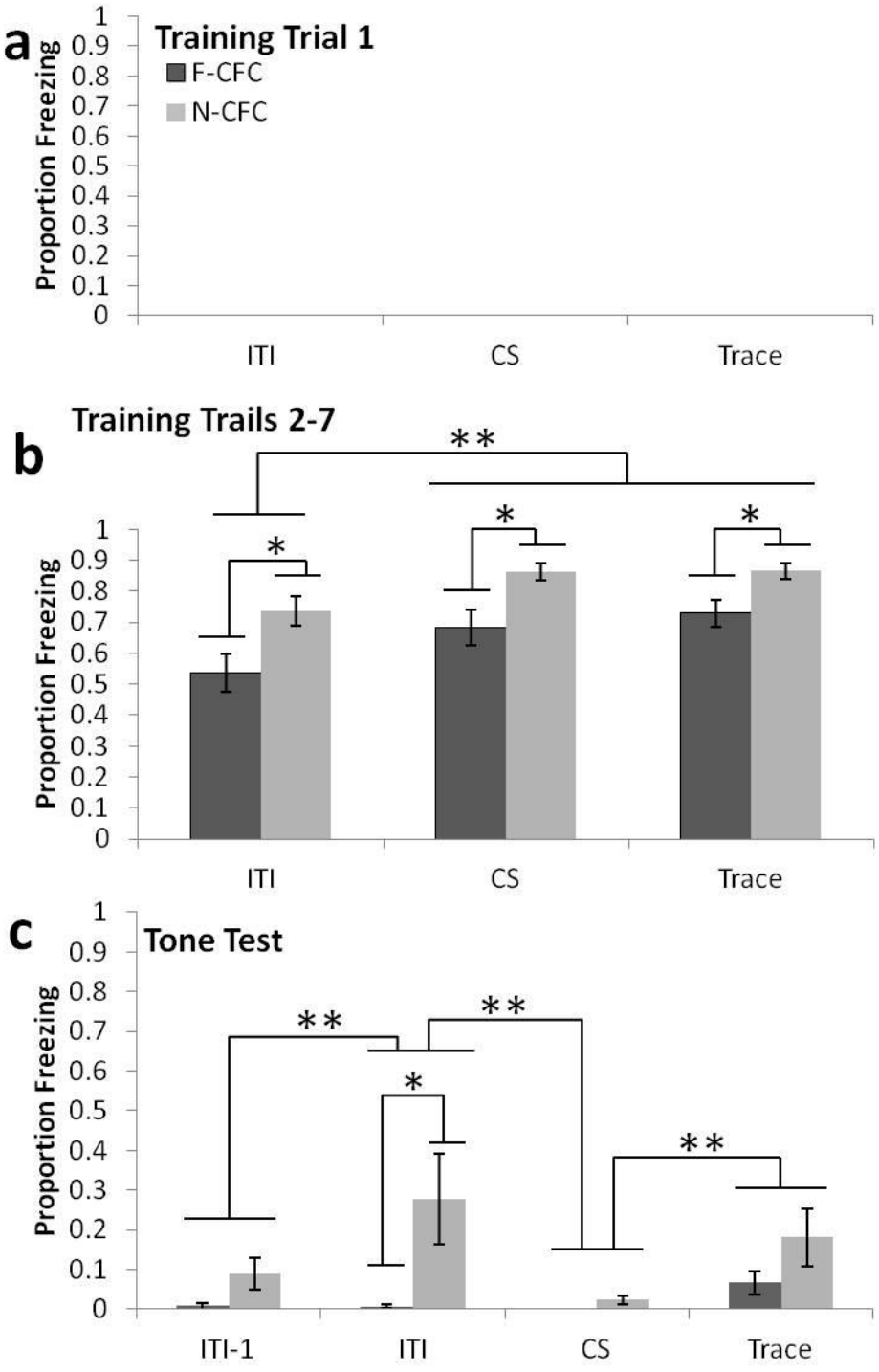

Figure E4-1: Mean +/- SEM proportion of trial period freezing for F-CFC and N-CFC subjects during contextual fear conditioning and tone CS testing. (a) Trial one of training in CFC. (b) Trials 2-7 of training in CFC with significantly more freezing for N-CFC subjects than F-CFC subjects but both groups demonstrated significantly more freezing during the CS and Trace intervals compared to the ITI

(p<0.001). Significant difference training context (*) and trail period (**) is denoted with asterisk. (c) Freezing to the tone CS 24 hours after CFC with significantly more freezing for N-CFC subjects compared to F-CFC subjects during the ITI but significantly less freezing during the CS for N-CFC subjects suggesting generalization of contextually-elicited fear to the novel testing context.

Delay fear conditioning

Analyses of behavioral data for subjects trained in DFC were identical to analyses of TFC except that there was no trace interval between CS offset and US onset during which to measure and compare freezing.

Training. Final sample sizes for training are F-DFC: n=10 and N-DFC; n=8. Two separate two-way repeated measures ANOVAs were conducted with training context (N-DFC, F-DFC) as the between subjects factor and trial period (ITI, CS) as the within subjects factor. For Trial 1 (Figure E4-2a) statistical analyses revealed there was no main effect of training context ($F_{(1,35)}$ = 0.867, p=0.366), no significant main effect of trial period ($F_{(1,35)}$ = 0.867, p=0.366), with no significant interaction between training context and trial period ($F_{(1,35)}$ = 1.133, p=0.303). There was little to no freezing for either N-DFC or F-DFC subjects during the first trial prior to the first US delivery.

For Trials 2-7 (Figure E4-2b) statistical analyses revealed there was no main effect of training context ($F_{(1,35)}$ = 4.065, p=0.061), no significant main effect of trial period ($F_{(1,35)}$ = 3.235, p=0.091), and no significant interaction between training context and trial period ($F_{(1,35)}$ = 0.0054, p=0.942). While there were no significant differences between N-DFC and F-DFC subjects, both demonstrated

substantial freezing across trial periods however there was a trend (p=0.061) toward more freezing during the CS.

Tone Testing. Final sample sizes for tone testing are F-DFC: n=5 and N-DFC: n=4. The data from ITI-1 during tone testing are interpreted as a “baseline” level of freezing to the novel testing context and are separated from the other ITI freezing data (Figure E4-2c). In order to determine if context novelty significantly effects freezing to the tone CS during testing, another two-way repeated measures ANOVA was conducted with training context (N-DFC, F-DFC) as the between subjects factor and trial period (ITI-1, ITI, CS) as the within subjects factor. Statistical analyses revealed there was no significant main effect of training context ($F_{(1,26)}$ = 0.00007, p=0.994), a significant main effect of trial period ($F_{(2,26)}$ = 10.109, p=0.002), with no significant interaction between training context and trial period ($F_{(2,26)}$ = 0.0321, p=0.969). SNK *post hoc* analysis identified significant differences during ITI-1 and both the CS (p =0.003) and ITI (p=0.003) trial periods. There were no differences in freezing between N-DFC and F-DFC subjects during tone testing and both groups demonstrated very little freezing during ITI-1 suggesting little to no generalization of fear to the novel testing context.

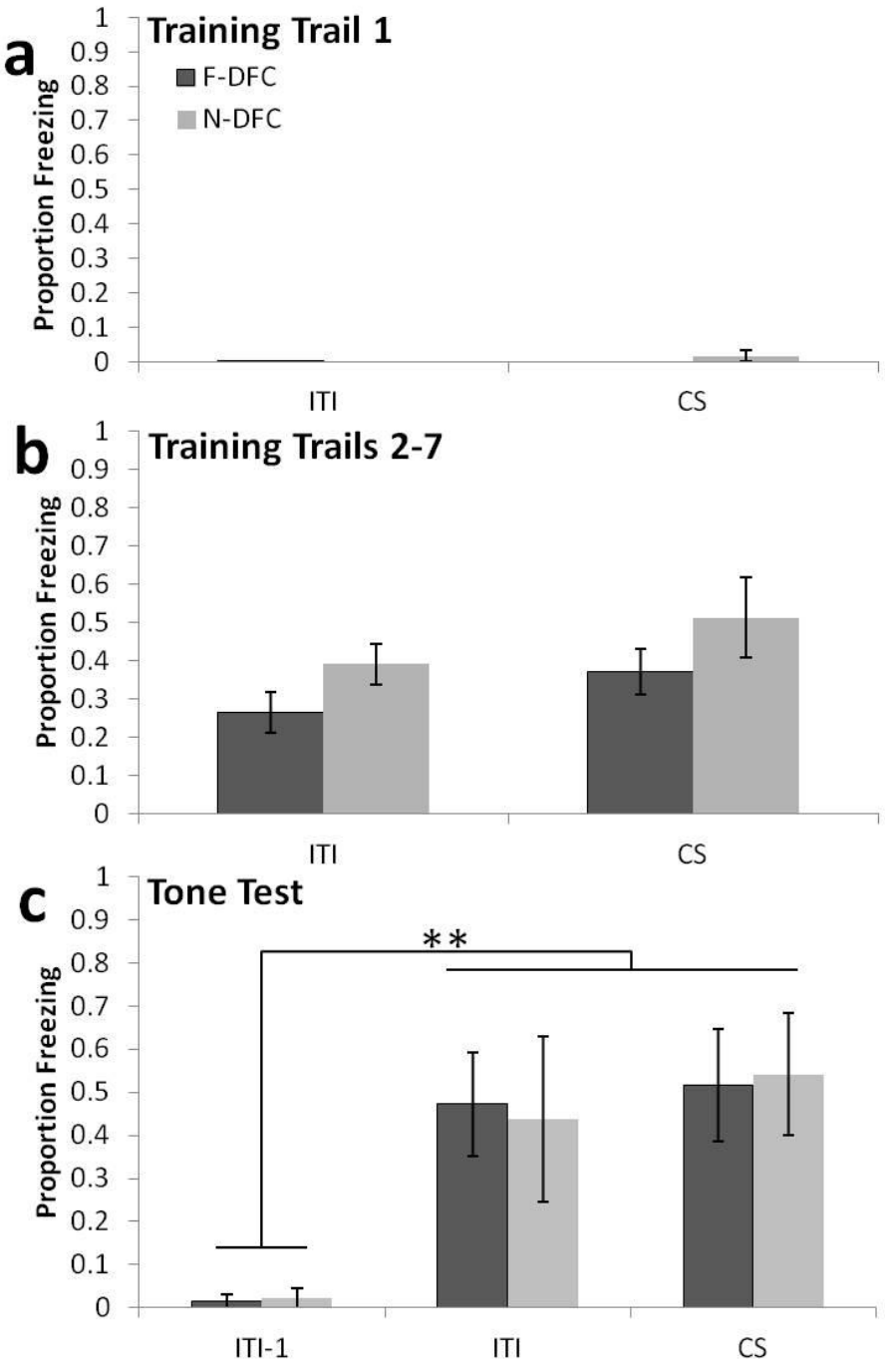

Figure E4-2: Mean +/- SEM proportion of trial period freezing for F-DFC and N-DFC subjects during delay fear conditioning and tone CS testing. Significant difference between groups (*) and trail period (**) is denoted with asterisk (a) Trial one of training in DFC. (b) Trials 2-7 of training in DFC with a trend toward more freezing during the CS (p=0.061). (c) Freezing to the tone CS 24 hours after training in DFC with significantly less freezing during ITI-1 than other

periods suggesting little to no generalization of contextually-elicited fear to the novel testing context.

Immunohistochemical examination of regional patterns of Arc expression

Hippocampal neuronal cells stained positive for Arc protein expression were quantified across the septotemporal and transverse axes of both dorsal and ventral hippocampus. Statistical analyses are reported for Arc positive cell counts/mm^2 for subjects trained in either contextual or delay fear conditioning in a familiar or novel context. Arc protein expression is compared to the combined twelve HC subjects across Experiments 2-4. Both differences between subfields and experimental groups are reported however differences between subfields will be deemphasized here and will be discussed fully in the next section of the manuscript.

Arc positive cell counts for subjects trained in DFC were averaged across two independent counters instead of three. CFC and DFC Arc expression data are analyzed separately. Arc protein expression data from one subject trained in DFC in a novel context was not included due to failure to deliver the footshock US during training. Final sample sizes were F-CFC: n=5, N-CFC: n=5; F-DFC: n=5, N-DFC: n=4, HC: n=12.

Contextual fear conditioning

Arc positive cells per mm^2 in dorsal and ventral hippocampal subfields CA1 & CA3 compared to home cage control subjects. In order to determine whether context pre-exposure significantly affected Arc protein expression following CFC,

a two-way repeated measure ANOVA was conducted on dorsal hippocampus immunohistochemical data (Figure E4-3a) with training context (F-CFC, N-CFC, HC) as the between subjects factor and hippocampal subfield (CA1, CA3) as the within subjects factor. Statistical analyses revealed there was a significant main effect of training context ($F_{(2,43)}$ = 13.44, p<0.001), a significant main effect of subfield, ($F_{(1,43)}$ = 75.485, p<0.001), with no significant interaction between training context and subfield, ($F_{(2,41)}$ = 2.342, p=0.123). SNK *post hoc* analysis identified significant enhancements in Arc protein expression for F-CFC and N-CFC subjects relative to HC controls (p<0.001, p=0.002, respectively). Enhancement relative to HC Arc protein expression, particularly in CA1 was not as strong when comparing enhancement to the three HC subjects specific to this experiment instead of all twelve subjects. Arc protein was enhanced in subjects trained in CFC relative to HC subjects across subfields, yet there were no significant differences in protein expression between subjects trained in a familiar versus novel context (p=0.998).

A separate two-way repeated measure ANOVA was conducted on ventral hippocampus immunohistochemical data (Figure E4-3b) with training context (F-CFC, N-CFC, HC) as the between subjects factor and hippocampal subfield (CA1, CA3) as the within subjects factor. Statistical analyses revealed there was a significant main effect of training context ($F_{(2,43)}$ = 9.154, p=0.002), a significant main effect of subfield, ($F_{(1,43)}$ = 54.249, p<0.001), and a significant interaction between training context and subfield, ($F_{(2,43)}$ = 7.002, p=0.005). SNK *post hoc* analysis identified that Arc protein was significantly enhanced relative to HC

subjects for F-CFC and N-CFC subjects but only in ventral CA1 (p<0.001). Arc protein was enhanced in subjects trained in CFC relative to HC subjects in ventral CA1 and not CA3 however there were again there were no significant differences in protein expression between subjects trained in a familiar versus novel context.

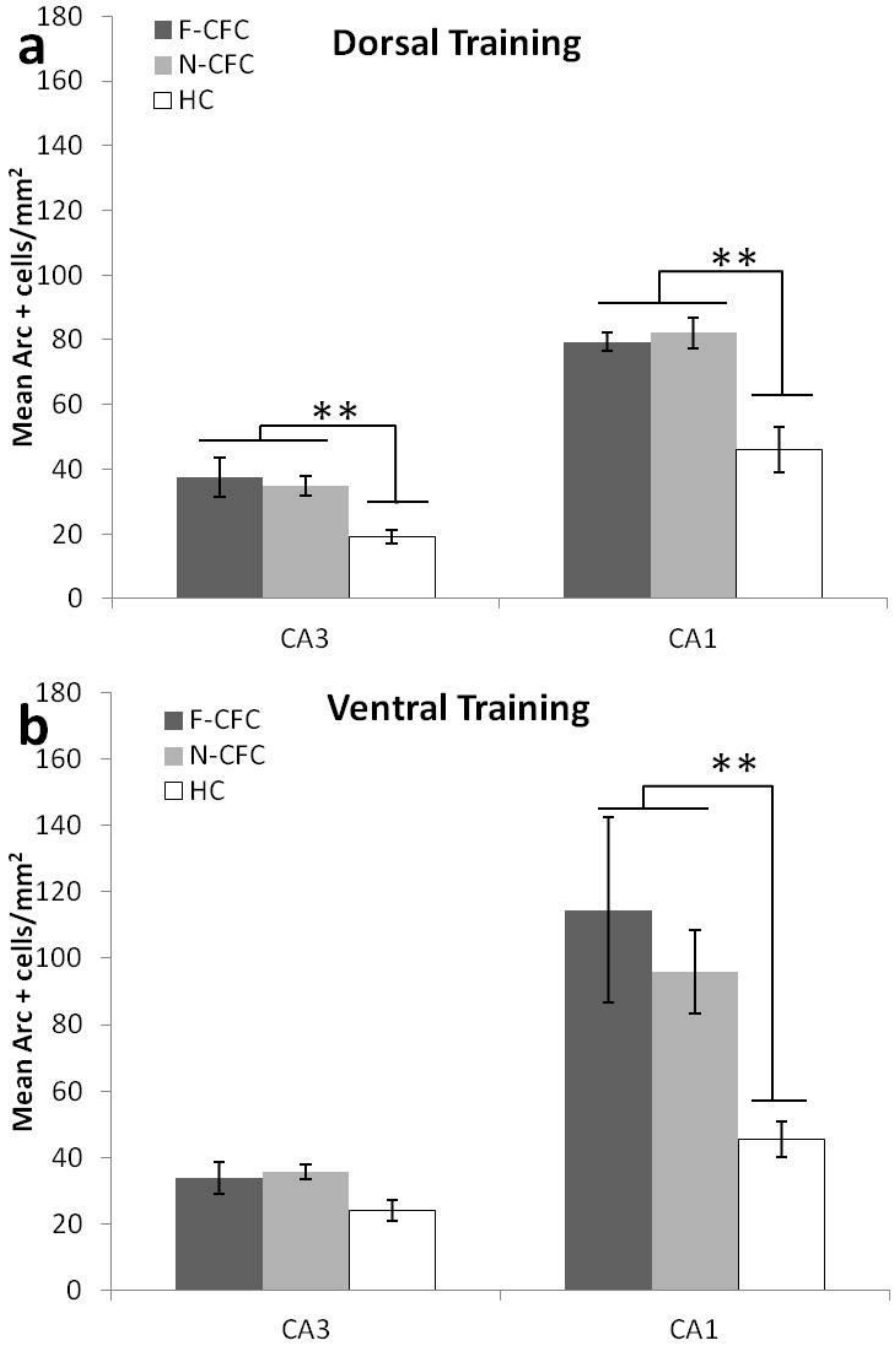

Figure E4-3: Arc positive cells counts per mm^2 in CA3 and CA1 subfields in dorsal and ventral hippocampus. Significant difference between subfields (*) and groups (**) is denoted with asterisk (a) Mean ± SEM Arc positive cells per mm^2 in the dorsal hippocampus was significantly enhanced in F-CFC and N-CFC subjects relative to HC controls ($p<0.001$, $p=0.002$, respectively). (b) Mean ± SEM Arc positive cells per mm^2 in the ventral hippocampus was significantly

enhanced relative to HC subjects for F-CFC and N-CFC subjects but only in CA1 (p<0.001).

Delay fear conditioning

Arc positive cells per mm^2 in dorsal and ventral hippocampal subfields CA1 & CA3 compared to home cage control subjects. In order to determine whether context pre-exposure significantly affected Arc protein expression following delay fear conditioning compared to HC control subjects a two-way repeated measure ANOVA was conducted on dorsal hippocampus immunohistochemical data (Figure E4-4a) with training context (F-DFC, N-DFC, HC) as the between subjects factor and hippocampal subfield (CA1, CA3) as the within subjects factor. Statistical analyses revealed there was a significant main effect of training context ($F_{(2,41)}$ = 5.883, p<0.011), a significant main effect of subfield, ($F_{(1,41)}$ = 50.135, p<0.001), with no significant interaction between training context and subfield, ($F_{(2,41)}$ = 0.792, p=0.468). SNK *post hoc* analysis identified significant enhanced in Arc protein for F-DFC and N-DFC subjects relative to HC controls (p<0.018, p=0.027, respectively). Enhancement relative to HC Arc protein expression, particularly in CA1 was not as strong when comparing enhancement to the three HC subjects specific to this experiment instead of all twelve subjects. Arc protein was enhanced in subjects trained in DFC relative to HC subjects across subfields yet there were no significant differences in protein expression between subjects trained in a familiar versus novel context (p=0.692).

A separate two-way repeated measure ANOVA was conducted on ventral hippocampus immunohistochemical data (Figure E4-3b) with training context (F-DFC, N-DFC, HC) as the between subjects factor and hippocampal subfield (CA1, CA3) as the within subjects factor. Statistical analyses revealed there was a significant main effect of training context ($F_{(2,41)}$ = 12.689, p<0.001), a significant main effect of subfield, ($F_{(1,41)}$ = 74.135, p<0.001), and a significant interaction between training context and subfield, ($F_{(2,41)}$ = 9.542, p=0.001). SNK *post hoc* analysis identified Arc protein was significantly enhanced relative to HC subjects for F-DFC and N-DFC subjects but only in ventral CA1 (p<0.001). Arc protein was enhanced in subjects trained in DFC relative to HC subjects in ventral CA1 and not CA3 however there were again no significant differences in protein expression between subjects trained in a familiar versus novel context.

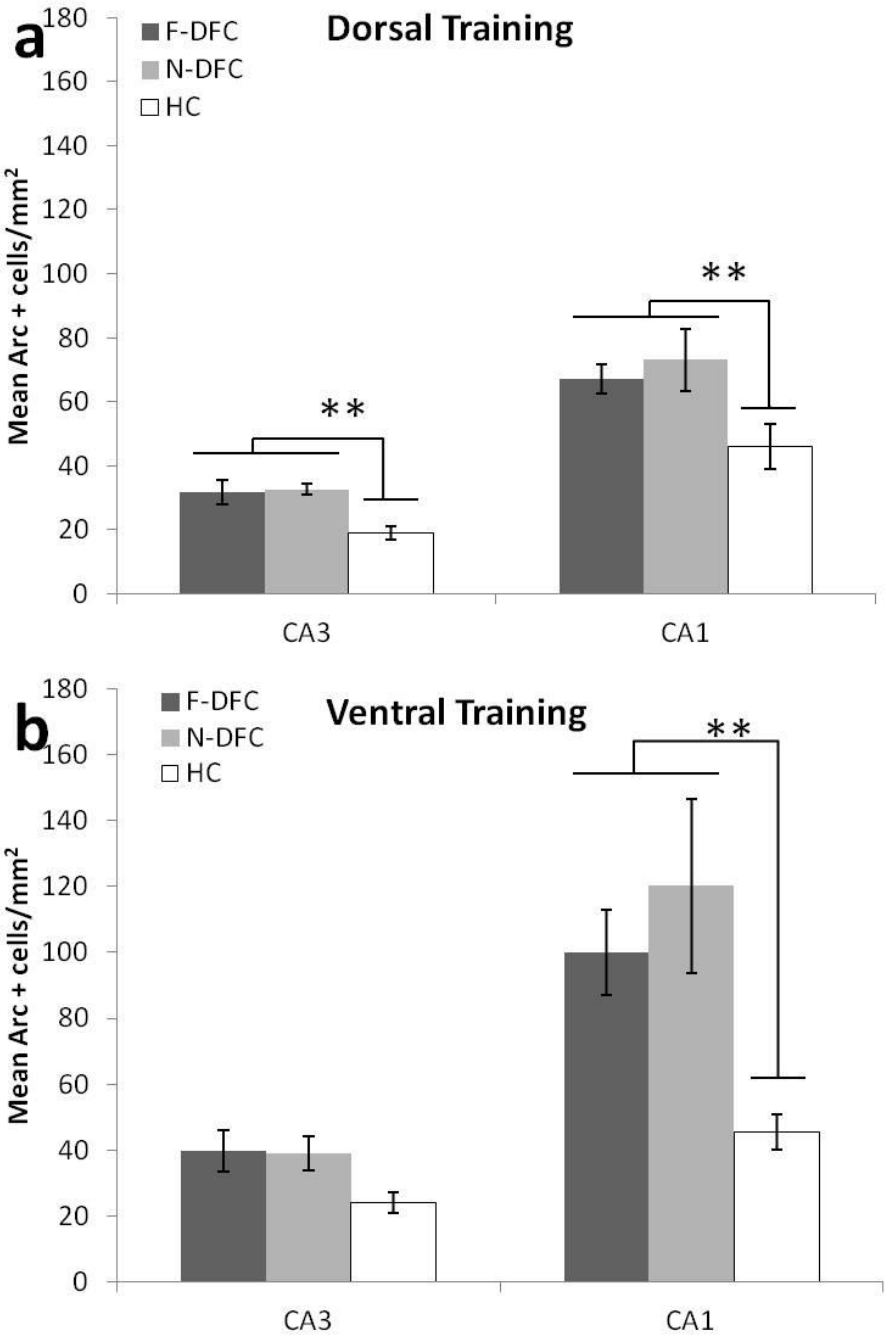

Figure E4-4: Arc positive cells counts per mm^2 in CA3 and CA1 subfields in dorsal and ventral hippocampus. Significant difference between subfields (*) and groups (**) is denoted with asterisk (a) Mean ± SEM Arc positive cells per mm^2 in the dorsal hippocampus was significantly enhanced in F-DFC and N-DFC subjects relative to HC controls (p=0.018, p=0.027, respectively). (b) Mean ± SEM Arc positive cells per mm^2 in the ventral hippocampus was significantly

enhanced relative to HC subjects for F-DFC and N-DFC subjects but only in CA1 (p<0.001).

Discussion

The current experiment sought to determine whether the patterns of Arc protein enhancement in dorsal and ventral CA3 following TFC are unique to context-US or explicit CS-US components of conditioning, and to determine whether context pre-exposure has a similar effect on either behavior or Arc protein enhancement as it did in Experiment 3. Subjects trained in contextual and delay fear conditioning were expected to demonstrate unique patterns of Arc protein enhancement relative to HC control subjects. Context pre-exposure was expected to affect conditioned responding during trained in CFC but not DFC. After discussing the individual results of this experiment these data will then be directly compared to Experiment 2 and 3 in Chapter 4.

Behavioral analysis of contextual fear conditioning versus delay fear conditioning

Context pre-exposure reduced conditioned responding during training for subjects trained in contextual but not delay fear conditioning

Subjects trained in CFC in a familiar context (F-CFC) were expected to demonstrate reduced conditioned responding during training, as the context is directly paired with the US. Consistent with our hypothesis, F-CFC subjects showed significant reductions in freezing throughout the training session compared to N-CFC subjects. This difference was not observed for DFC

subjects. This is consistent with prior research in which context pre-exposure was used as a control procedure for explicit CS pre-exposure (Escobar et al., 2002). Since our pre-exposure procedure does not include the tone CS which is later paired with the US during DFC, we did not expect to see an effect of context pre-exposure in the training data. However, while there was a significant effect of pre-exposure on freezing behavior for subjects trained in CFC and not DFC, both groups showed a significant, or trend toward significant, increase in freezing during the temporal interval most closely preceding the US. This is consistent with the observation that DFC subjects show greater freezing, though not significantly so, during the CS, which is the stimulus directly preceding and overlapping with the US. Similarly, subject trained in CFC, in either a novel or familiar context, demonstrated significantly more freezing during both the CS and trace periods compared to the ITI. Recall that during CFC no explicit conditioned stimuli are presented, so the “CS” and “trace” intervals are simply the time periods most closely preceding the US. This suggests that while pre-exposure had an effect of lowering the overall freezing response in F-CFC subjects, temporal precision of the response was unaffected.

Subjects trained in contextual fear conditioning, but not delay fear conditioning, demonstrated generalized contextually-elicited fear

Both CFC and DFC subjects were tested for tone elicited fear in a novel context. While this test is typically carried out to assess the associative strength of the explicit CS-US contingency, both groups were tested for reasons outlined above. Tone testing in a novel context serves a dual function. First, using a CS

testing procedure which mimics the temporal structure of training but in a novel context allows for the separation of the first ITI from remaining ITIs and trial periods. This allows us to dissociate conditioned responding initially elicited by the novel testing context from other events (explicit or temporal) occurring during tone testing. DFC subjects showed very little freezing during ITI-1 suggesting very little to no generalization of fear to a novel context regardless the context familiarity during training. By contrast, N-CFC subjects demonstrated significantly more freezing during the ITI than did F-CFC subjects, suggesting enhanced generalization of fear to a novel context when the initial training in CFC also occurs in a novel context.

Additionally, during the tone test N-CFC subjects froze significantly more during the ITI and trace intervals than during the CS ($p<0.001$, $p=0.020$, respectively). N-CFC subjects actually showed the lowest levels of freezing during CS presentation all suggesting that the novel context itself, not the CS, is eliciting fear. This is obviously not the case for subjects trained in DFC who show substantial freezing during the CS (Figure E4-2b). However we did not see a similar trend toward more freezing to the CS compared to the ITI like during training. In summary, subjects receiving CFC in a novel context demonstrated more freezing during training which generalized to a novel context. This same generalization was not observed for subjects trained in a familiar context nor was it observed for subjects trained in DFC regardless of context familiarity.

Arc protein expression

Subjects trained in CFC or DFC demonstrated significant enhancements in Arc protein in CA1 and CA3 of the dorsal hippocampus and CA1 of the ventral hippocampus relative to HC control subjects. Significant reductions in Arc protein expression following conditioning in a familiar context were expected for subjects trained in CFC and not DFC, however no such effect was observed.

Contextual fear conditioning

Dorsal hippocampus Arc protein expression is enhanced in both CA3 and CA1 relative to home cage control subjects. Subjects trained in CFC were expected to show significant enhancements in Arc protein relative to HC subjects across dorsal CA1 and CA3. Dorsal hippocampus CA1 and CA3 are both critically necessary for contextual fear conditioning (Hunsaker & Kesner, 2008), and our results extend this observation in that Arc protein is significantly enhanced within both regions following CFC. However, as stated previously, if we only compare to Arc protein expression data from the 3 HC subjects who were sacrificed and underwent tissue processed at the same time as the rest of the subjects trained in CFC, then the learning induced differences in Arc protein expression in CA1 is minimized. See Experiment 2 for justification for using the 12 HC subjects.

Both dorsal CA1 and CA3 are implicated in contextual fear conditioning, and both activity within these regions and Arc expression reflect the processing of spatial and contextual elements of some experiences (Vazdajanova and Guzowski, 2004; Guzowski 1999). Thus the finding that Arc protein expression within these regions was unaffected by context familiarity was unexpected. The

lack of an effect of context familiarity is particularly surprising given the significant reduction in conditioned responding observed for subjects trained in a familiar context reported above. This suggest that Arc protein expression in these regions, while necessary for learning context-US associations (Czerniawski et al., 2011), is not necessarily sensitive to experiences which modulate contextual fear learning. Thus the role of Arc protein in supporting contextually elicited fear in the dorsal hippocampus may more overlap with its putative role in general spatial learning (Czerniawski et al., 2009; Vazdarjanova et al., 2006; Ramirez-Amaya et al., 2005).

Another reason dorsal CA1 and CA3 Arc protein expression may have been unaffected by context pre-exposure is that while freezing was significantly reduced for F-CFC subjects, the temporal precision of the response was not significantly altered. Even though subjects in a familiar context froze less, they still demonstrated significant increases in freezing during trial periods closest to the US. This suggests that while the amount of contextually-elicited fear is reduced, the temporal precision of responding, which may also be supported hippocampal activity (Eichenbaum et al., 2016) specifically in dorsal CA1 (Hoge & Kesner, 2007), is unaffected by context pre-exposure.

Ventral hippocampus Arc protein expression is enhanced in CA1 relative to home cage control subjects. Within the ventral hippocampus, a significant enhancement in Arc protein was observed in CA1 following CFC but not DFC. Enhancement in this subfield was expected as ventral CA1 has dense reciprocal connections to the amygdala and provides the main route by which information

from the amygdala is linked to contextual representations in the dorsal hippocampus, as outlined in the General Introduction. Similar to the patterns observed in dorsal hippocampus, there was no effect of context familiarity even though conditioned fear was significantly reduced for subjects trained in a familiar context. However, as discussed above, this assumption that a procedure that produces contextual latent inhibition would reduce Arc protein is not supported by the data.

Additionally, there is again no additive effect on Arc protein due to both novel context exposure and fear conditioning. N-CFC subjects do not show enhancement relative to F-CFC subjects, which would be expected if Arc protein due to fear conditioning occurred in addition to Arc induced by novel context exposure. For CFC subjects, Arc protein expression in the ventral hippocampus was enhanced in CA1 and not CA3. Others have reported a role for ventral CA3 in the retrieval of contextual associations following fear conditioning (Hunsaker & Kesner, 2008); however the patterns of Arc expression reported here were identified following training and are more likely to reflect encoding of contextual associations rather than retrieval.

Delay fear conditioning

Dorsal hippocampus Arc protein expression is enhanced in both CA3 and CA1 relative to home cage control subjects. Subjects trained in DFC were expected to show significant enhancement in Arc protein in both CA3 and CA1, as conditioning to an explicit CS also produces contextual conditioning. However,

Arc protein expression was expected to be unaffected by context pre-exposure during training as no effect was observed in subjects trained in TFC (Experiment 3) and acquisition of the explicit CS-US contingency is unaffected by blocking Arc protein (Czerniawski et al., 2011). Additionally, the documented effects of context pre-exposure (Fanselow, 1986; Fanselow, 1990) and the explicit role of the hippocampus in CFC (Fanselow and Poulos, 2005; Maren, 2001) do not apply to DFC. The present data support both of these hypotheses. Arc protein was significantly enhanced in CA1 and CA3 following conditioning and likely supports contextual learning since blocking Arc produces a reduction in contextually-elicited fear (Czerniawski et al., 2011), but has no effect on learning the explicit CS-US contingency.

Also consistent with our predictions, Arc protein expression was not significantly altered due to training a familiar versus novel context. This was predicted based on this same lack of an effect for subjects trained in TFC. Additionally, context pre-exposure is used as a control procedure for explicit CS latent inhibition (Escobar et al., 2002) because of its lack of impact of responding which provides further support for the lack of an effect on Arc protein enhancement. Hence Arc protein expression in the dorsal hippocampus following DFC in not affected by context pre-exposure and likely reflects hippocampal dependent context-US components of the fear conditioning task. These conclusions will be further supported by direct comparisons to subjects trained in CFC and TFC relating the magnitude of contextually elicited fear during context testing with the amount of dorsal CA3 and CA1 enhancement following training.

Ventral hippocampus Arc protein expression is enhanced in only CA1 relative to home cage control subjects. Arc expression in the ventral hippocampus was expected to be minimal following training because the hippocampus is not necessary for learning delay fear conditioning, and ventral hippocampus is thought to be more involved in the retrieval of contextual fear rather than encoding (Hunsaker & Kesner, 2008). While the present data partially support this hypothesis, Arc protein was significantly enhanced in ventral CA1. However, Arc protein expression in ventral CA1 following DFC likely does not reflect explicit CS-US processing as Arc is similarly enhanced following CFC and novel context exposure (Experiment 2) both of which preclude the possibility Arc protein in ventral CA1 reflecting an explicit CS-US contingency. Ventral CA1 Arc protein enhancement in subjects trained in DFC is also not likely reflecting CS-US processing as preventing Arc protein in this region has no effect on acquisition of the CS-US associations in delay fear conditioning (Czerniawski et al. 2011). While only ventral CA1 is enhanced following training in DFC, it remains to be seen if these same subfields will show enhancement following testing for contextually-elicited fear. Those data will be reviewed in Chapter 5.

Conclusion

Subjects trained in CFC and DFC demonstrated similar patterns of Arc protein enhancement relative to HC subjects even though CFC, and not DFC subjects, showed evidence of a behavioral effect of context pre-exposure during both training and tone testing. Arc protein was significantly enhanced across both CA3 and CA1 subfields in the dorsal hippocampus and within CA1 of the ventral

hippocampus following conditioning in either task. Arc protein in dorsal CA3 and possibly CA1 may reflect contextual components of fear conditioning tasks as both CFC and DFC produce contextually elicited fear. Ventral CA1 provides the main pathway by which information from the amygdala reaches the dorsal hippocampus, however Arc expression in this subfield likely does not reflect explicit components (CS-US, context-US, or otherwise) of learned associations. Arc protein in ventral CA1 is similarly enhanced following novel context exposure (Experiment 2), however these data need to be directly compared to the data from Experiment 2 and subjects trained in TFC (Experiment 3) to provide further support for this hypothesis. This is included in the next section.

Chapter 4: Comparison across Experiments

As shown in Experiment 2 and 3, after controlling for the influence of context novelty, subjects trained in TFC demonstrated enhancements in Arc protein in dorsal CA3 and ventral CA1 and CA3 of the hippocampus. By contrast, in Experiment 4 the patterns of expression observed for subjects trained in CFC and DFC both showed significant enhancements in Arc protein in dorsal CA3 and CA1 and ventral CA1. The common enhancement in Arc protein in dorsal CA3 across groups may reflect the contextual components of fear conditioning as subjects trained in each of our 3 fear conditioning paradigms (TFC, CFC, DFC) demonstrate contextually elicited fear. Significant enhancement in Arc protein in ventral CA1 was also observed for subjects trained in fear conditioning but also following novel context exposure, suggesting enhancement in this subfield may occur independently from fear conditioning. However, significant enhancements in ventral CA3 seem unique to subjects trained in TFC and may reflect the explicit tone CS components of the trace fear conditioning paradigm, as reviewed in Experiment 3. Given these individual patterns, a direct comparison of Arc protein enhancement elicited by novel context exposure as well as by the different fear conditioning tasks is still required to determine whether enhancements within given subfields are unique to trace fear conditioning.

Along with a comparison of Arc protein expression, the effect of context pre-exposure on conditioned responding to the explicit tone CS for subjects trained in TFC and DFC will be compared, as well as differences in contextually elicited fear for all three fear conditioning task. As discussed in Experiment 3, the

explicit tone CS for subjects trained in TFC may serve a different function compared to DFC. Subjects trained in TFC demonstrated significantly reduced responding during presentation of the CS during the tone test and responding was further reduced, though not significantly, for subjects trained in a familiar context. The same pattern was not observed for subjects trained in DFC where freezing to the CS not different than other trial periods. Additionally, CS-US learning for subjects trained in TFC is significantly impaired by blocking Arc protein expression in the dorsal hippocampus while CS-US learning in delay conditioning is not (Czerniawski et al., 2011) suggesting that Arc protein dependent contextual representations in the dorsal hippocampus may interact with CS-US learning in TFC but not DFC. Given the role of Arc protein in the dorsal hippocampus supporting both contextual learning and trace fear conditioning subjects trained in TFC may be particularly sensitive to context pre-exposure compared to subjects trained in DFC especially. The behavioral analyses of freezing during tone testing described in Experiments 3 and 4 suggest that there is no effect of context pre-exposure for TFC or DFC subjects; however a cross-experiment comparison may highlight an interaction between type of fear conditioning and context pre-exposure. For subjects trained in CFC, context pre-exposure significantly affected freezing during tone testing however these subjects were not included in the comparison of CS elicited freezing because, as discussed in Experiment 4, freezing was likely not CS-elicited fear as an explicit CS was not presented during training.

We previously reported robust contextual latent inhibition as a result of context pre-exposure for subjects trained in a familiar context for all three fear conditioning task however statistical analyses were not reported. These analyses will be reviewed in this section by comparing contextually elicited fear between subjects trained in a novel versus familiar context and between the three different types of fear conditioning. Differences in contextually elicited fear between groups are expected particularly for subjects trained CFC as the context is the only stimulus paired with the US. Standard interpretations of associative theory (Rescola & Wagner 1972) predict that the explicit tone CS presented during training for TFC and DFC will share some of the associative strength normally ascribed to the context producing less contextually elicited responding.

Additionally, differences in contextually elicited responding observed during the context test may be reflected in initial differences in dorsal hippocampal Arc protein induced by training. As reported above Arc enhancement in dorsal CA3 and likely CA1 may reflect processing of contextual representations (context-US) supporting contextually elicited fear. Research from our lab has shown that blocking Arc protein in the dorsal hippocampus prior to training produces a deficit in contextual learning across different fear conditioning tasks (Czerniawski et al., 2011) even for subjects trained in DFC for which blocking Arc protein has no effect on explicit CS-US learning.

All data selected for statistical analyses in this section were individually reported in the previously described experiments (2-4), except the behavioral data during context testing. The present analysis is three-fold. First, a behavioral

analysis of the effect of context pre-exposure on freezing behavior elicited by the explicit CS for subjects trained in TFC and DFC will provide a better understanding of the potentially conflicting functional role of the CS. Additionally, a second behavioral analysis to demonstrate contextual latent inhibition and directly compare contextually elicited fear across all trained groups. Finally, a third analysis of Arc protein expression within select groups, across Experiments 2-4, will permit a determination of whether there was a subfield-specific enhancement, unique to fear conditioning, reflecting CS-US and/or context-US processing.

Methods

Behavioral data and Arc protein expression data from Experiments 2-4 were directly compared to explore significant differences in CS and contextually elicited freezing and significant differences between subfields and across experimental groups.

Procedure

The procedures from which these data were taken are described in the General Methods as well as the methods sections from Experiments 2-4.

Subjects. Behavioral data from the tone test will be compared to determine whether context pre-exposure has a differential effect on fear conditioned to the explicit tone CS for TFC (F-TFC: n=5, N-TFC: n=5) and DFC (F-DFC: n=5, N-DFC: n=4) subjects. Additionally, contextually-elicited freezing during the context test will be compared for all groups trained in fear conditioning to assess

differences in contextually-elicited fear and contextual latent inhibition (F-TFC: n=5, N-TFC: n=5; F-CFC: n=10, N-CFC: n=9; F-DFC: n=5, N-DFC: n=4).

Groups selected for the cross experiment analysis of Arc protein expression include the novel context exposure group (n=5) (Experiment 2), subjects trained in TFC in a familiar context (n=5) (Experiment 3), and subjects trained in both CFC (n=5) and DFC (n=5) in a familiar context (Experiment 4). These groups were selected so that Arc protein expression induced by novel context exposure could be directly compared to Arc induced by training in our three fear conditioning task in a familiar context.

Data transformations

All Arc protein expression data included in these analyses was transformed into percent increase in Arc protein expression relative to HC levels. This transformation is described and justified in the General Methods. Briefly, this transformation allows for the normalization of novel context exposure/ fear conditioning induced Arc protein to baseline levels of Arc protein observed across 12 HC subjects. This allows for a direct comparison of different groups within subfields but importantly allows for more meaningful comparisons of differences in Arc protein between different subfields of different sizes and different cell packing densities (Pyapali et al., 1998). Direct comparison of training induced Arc protein expression can be misleading (see Experiment 1 analyses) as significant differences between subfields is influenced by initial baseline differences observed in HC subjects.

Results

Comparisons of freezing during tone testing for TFC and DFC subjects were conducted to first determine whether we replicated the common observation of greater CS freezing following DFC compared to TFC, and second to determine whether context pre-exposure differentially affected CS-US learning in these groups. Recall that one potential effect of latent inhibition resulting from context-pre exposure is a shifting of associability toward the novel, non-pre-exposed stimulus, which in this case would be the CS. Contextually-elicited responding during context testing for subjects trained in all three fear conditioning are also compared to explore any differences in freezing due to the type of conditioning as well as effects of pre-exposure producing contextual latent inhibition. Additionally, we compared Arc protein expression induced by our 3 fear conditioning paradigms in a familiar context relative to that induced by novel context exposure. Subjects trained in fear conditioning in a novel context are not included in these analyses as Arc protein expression due to exposure to the novel training context cannot be dissociated from Arc protein induced by the fear conditioning task.

Cross experiment behavioral analysis

Behavioral comparisons across subjects conditioned to the explicit tone CS

Subjects were trained in TFC or DFC using an explicit tone as the CS. In order to explore differences in freezing for subjects trained in DFC and TFC, as well as different effects of context pre-exposure, CS-elicited freezing during the

tone test (Figure E5-1) was compared using a two-way ANOVA with training (DFC,TFC) and context (familiar, novel) as different factors. Statistical analyses identified a significant main effect training ($F_{(1,18)}$ = 5.633, p=0.031), no significant main effect of context ($F_{(1,18)}$ = 1.117, p=0.307), and no significant interaction between training and context ($F_{(1,18)}$ = 0.726, p=0.408). Freezing was significantly greater for subjects trained in DFC compared to TFC and while there was not a significant interaction only the subjects trained in TFC in a familiar context showed a substantial reduction in responding compared to other groups.

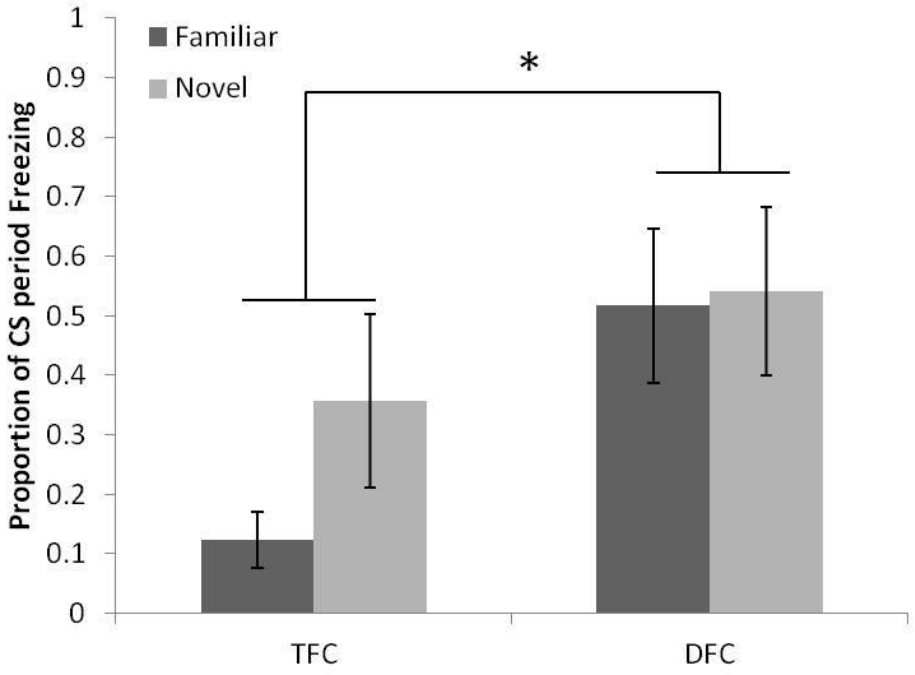

Figure E5-1: Proportion of time freezing during 3 CS presentations during tone testing for subjects trained in either TFC or DFC in a novel or familiar context. Freezing is significantly greater for DFC subjects compared to TFC subjects (p=0.031) and while there was not a significant interaction, freezing is particularly lower for subjects trained in TFC in a familiar context.

Contextually-elicited fear during trace, contextual and delay fear conditioning for subjects trained in a familiar or novel context

Subjects trained in TFC, CFC, or DFC in a familiar or novel context were tested for contextually elicited fear in their original trained context. In order to test for contextual latent inhibition in subjects trained in a familiar context the proportion of the session spent freezing was compared for all groups trained in a fear conditioning task; these data are illustrated in Figure E5-2. A two-way ANOVA with training (TFC,CFC, DFC) and context (familiar, novel) as factors identified no significant main effect training ($F_{(2,37)}$ = 1.80, p=0.182), a significant main effect of context ($F_{(1,37)}$ = 12.897, p=0.001), with no significant interaction between training and context ($F_{(2,37)}$ = 0.176, p=0.839). As expected, these data suggest that pre-exposure to the training context resulted in robust contextual latent inhibition across all three fear conditioning tasks.

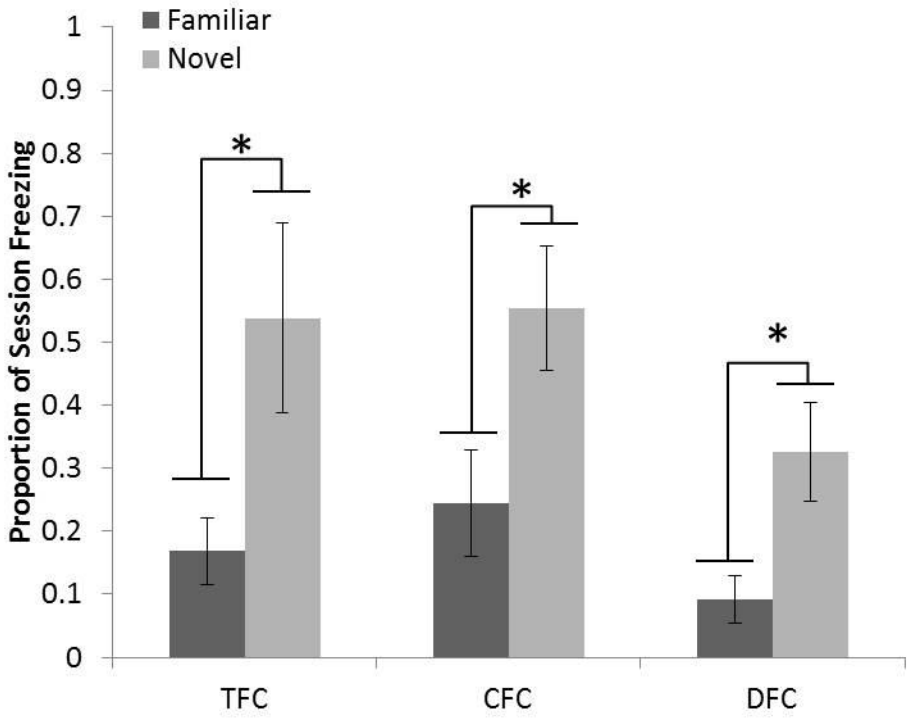

Figure: E5-2: Proportion of session freezing for subject trained in TFC, CFC, or DFC in a familiar or novel context during testing for contextually elicited fear. Freezing is significantly reduced for subjects trained in a familiar context (p=0.001) with no differences in freezing between groups.

Cross experiment analysis of Arc protein

Arc protein expression relative to home cage subjects across Experiments 2-4 in the dorsal hippocampus

In order to determine whether Arc protein enhancements in dorsal CA3 following trace fear conditioning is unique compared to either novel context exposure, other fear conditioning tasks, a two-way repeated measure ANOVA was conducted on immunohistochemical data (Figure E5-3a) with conditioning (F-TFC, F-CFC, F-DFC, N-NT) as the between subjects factor and hippocampal subfield (CA1, CA3) as the within subjects factor. Statistical analyses revealed

there was no main effect of conditioning ($F_{(3,37)}$ = 3.082, p=0.059), a significant main effect of subfield, ($F_{(1,37)}$ = 21.845, p<0.001), and a significant interaction between conditioning and subfield, ($F_{(3,37)}$ = 6.598, p=0.005). SNK *post hoc* analysis identified Arc protein enhancements to be significantly different between CA3 and CA1 but only for F-TFC subjects (p<0.001). Within dorsal CA3 F-TFC subjects' Arc protein was significantly greater than N-NT (p=0.004) and F-DFC subjects (p=0.029) and near significant compared to F-CFC subjects (p=0.052) while no such differences were observed in CA1. Comparing between groups Arc protein expression was similar across CA3 and CA1 except for subjects trained in TFC where Arc protein was significantly/near-significantly enhanced in dorsal CA3 compared to all other analyzed groups.

Arc protein expression relative to home cage subjects across Experiments 2-4 in the ventral hippocampus

In order to determine whether Arc protein enhancement in ventral CA3 following trace fear conditioning is unique compared to either novel context exposure, or other fear conditioning tasks, a two-way repeated measure ANOVA was conducted on immunohistochemical data (Figure E5-3b) with conditioning (F-TFC, F-CFC, F-DFC, N-NT) as the between subjects factor and hippocampal subfield (CA1, CA3) as the within subjects factor. Statistical analyses revealed there was no main effect of conditioning ($F_{(3,37)}$ = 1.427, p=0.274), a significant main effect of subfield, ($F_{(1,37)}$ = 23.235, p<0.001), and no significant interaction between conditioning and subfield, ($F_{(3,37)}$ = 0.804, p=0.511). Arc protein was significantly greater in ventral CA1 compared to CA3 and protein expression for

F-TFC subjects was not significantly greater than other groups. However, recall that while Arc protein in ventral CA1 was significantly enhanced relative to HC subjects for all trained/context exposed groups, only for F-TFC subjects was enhancement in ventral CA3 significantly different than HC subjects.

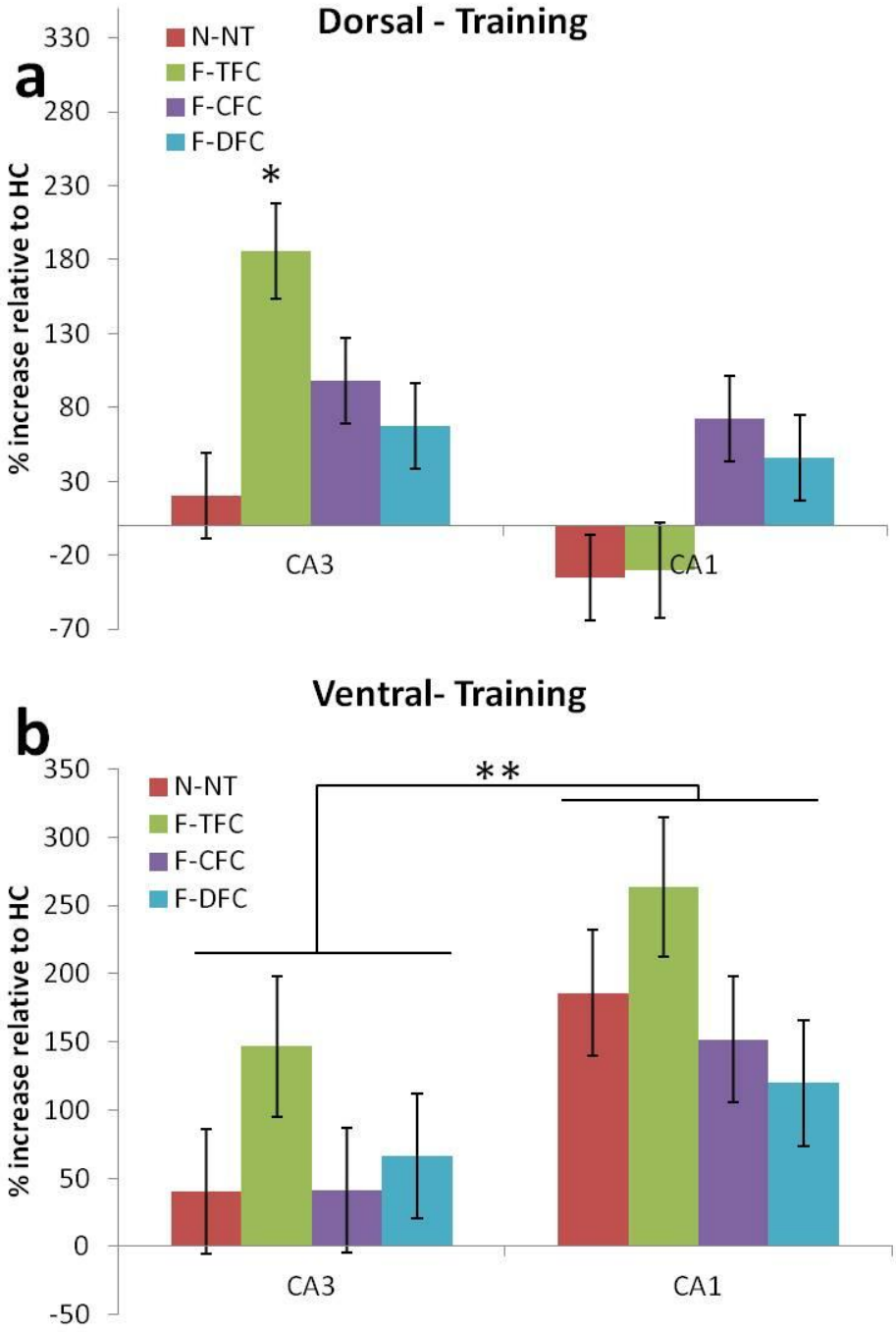

Figure E5-3: Mean+/-SEM percent increase in Arc protein relative to HC control subjects. (a) Dorsal hippocampal Arc protein expression within groups was similar across CA3 and CA1 except for F-TFC where Arc protein was significantly/near-significantly enhanced in dorsal CA3 compared to all other analyzed groups. (b) Ventral hippocampal Arc protein was significantly greater in CA1 compared to CA3 but protein expression for F-TFC subjects was not significantly greater than other groups; however only for F-TFC subjects was enhancement in ventral CA3 significantly different than HC subjects. Significant difference between groups (*) and subfields (**) is denoted with asterisk.

Discussion

There were three goals of the present series of analyses. First, behavioral data was compared across Experiments (2-4) to explore whether context pre-exposure differentially affected freezing to the explicit CS during tone testing. Second was to explore differences in contextually elicited freezing during context testing to determine whether latent inhibition is observed. Third, Arc expression induced by TFC was compared to that following novel context exposure, as well as CFC and DFC in order to explore whether TFC resulted in a unique subregional pattern of Arc expression.

Behavioral analysis of CS- and contextually-elicited fear

Differences in tone elicited fear due to context pre-exposure suggests different functional roles for the CS in delay and trace conditioning

Subjects trained in DFC froze significantly more than subjects trained in TFC. While this pattern is consistent with a large body of previous data (Bolles et al., 1978; Thrailkill & Shahan, 2014), there are differing accounts for why this occurs (discussed in length in the General Introduction and Experiment 1). Given

some of the issues with previous accounts, we proposed an alternative explanation for this difference in freezing to the CS by suggesting that the CS may serve a different function in TFC compared to DFC such that in TFC the CS does not predict the occurrence of the US but predicts the absence of US; in other words, it is possible that in TFC the CS is serving as a “safety signal”. This account is consistent with previous research demonstrating that varying the trace and ITI length within an appetitive trace conditioning task alters the function of the CS (Kaplan, 1984). As the length of the trace interval approaches the length of the ITI, instead of the CS eliciting an excitatory (approach) response, an inhibitory (withdrawal) response occurs (Kaplan, 1984). This suggests that, depending on the parameters of the conditioning task, a CS can serve different functions. Based on these observations, it is possible that the temporal parameters used in the trace fear conditioning procedure in our experiments alters the “meaning” of the CS such that CS may serve as a “safety signal” evoking a competing response to the freezing CR and hence freezing is reduced for TFC subject compared to DFC subjects. This stands in contrast to the standard transfer-of-strength based account of associative learning (Rescorla and Wagner, 1972) and instead suggests that TFC does not lead to a weaker CS-US contingency but produces a different CS-noUS contingency. Because these contingencies elicit different behaviors (CS-US elicits freezing while CS-noUS elicits movement) direct comparisons are problematic.

Using this interpretation we can better account for the behavior patterns observed in our data in general. First, while there was no significant effect of

context familiarity on freezing to the CS, there appears to be a trend in that direction for subjects trained in TFC (Figure E5-1); by contrast, for subjects trained in DFC, there is very clearly no effect of context familiarity. While there was not a significant interaction, a difference between DFC and TFC was only significant when training occurred in a familiar context. This observation is consistent with our interpretation of a different role for the CS in TFC. Here context familiarity, while having no effect on facilitating learning the explicit CS-US contingency in DFC subjects, appears to have a potential effect of facilitating the explicit CS-noUS contingency in TFC subjects. Less freezing to the CS for F-TFC subjects may reflect a facilitation of the CS-noUS contingency due to being trained in a familiar context.

During TFC, the CS is not directly paired with the US, and instead the context present during the trace interval is paired with the US. Due to the familiarity of the context during training for F-TFC subjects, the novel CS is a better predictor of when the US does/does not occur, enhancing contrast between the CS and context as predictive components of the task. However, for N-TFC subjects, both the context and CS are novel during training and hence both the context and CS have an equal history of occurrence with the US, promoting more generalized freezing across the trial periods. This differential effect of context familiarity is not observed in subjects trained in DFC because only the CS is paired with the US, hence pre-exposing to the context would have no effect on the explicit CS-US association.

These assumptions would then predict that for subjects trained in TFC it is specifically the acquisition of this CS-noUS contingency which is facilitating by context pre-exposure (contextual latent inhibition). As a further extension, if the primary difference between trace and delay conditioning is not one of associative strength, but one of what the CS predicts (US or noUS), then perhaps it is the successful acquisition of the noUS contingency that critically engages the hippocampus during trace fear conditioning which is simply not the association that is learned in delay conditioning. Additional behavioral and neurophysiological analyses would be needed to further verify this claim but that is beyond the scope of these studies. However, this interpretation does lend itself to accounting for differences in Arc protein expression within the hippocampus and will be revisited there.

Robust contextual latent inhibition was observed across fear conditioning tasks for subjects trained in a familiar context

Context pre-exposure was expected to produce contextual latent inhibition across fear conditioning task. For subjects pre-exposed to the context prior to fear conditioning, freezing was significant reduced during testing for contextually elicited fear demonstrating contextual latent inhibition. Subjects trained in all three fear conditioning tasks demonstrated contextual latent inhibition, but only for subjects trained in TFC did context pre-exposure also affect learning the explicit CS-US association, reported above.

Comparisons between CS-elicited and context-elicited fear and subfield-specific expression of Arc protein are discussed below. Based on previous research highlighting the critical role of the dorsal hippocampus for CFC, we initially expected that training subject in CFC would induce Arc protein to a greater extent than other tasks. However, given the effect of context pre-exposure on learning the explicit CS-US contingency for subjects train in TFC discussed in detail above, dorsal hippocampal Arc protein enhancement may be even more critical for acquisition and performance in TFC.

Arc protein expression enhancement reflecting CS-US and context-US processing

Differences in contextually elicited fear are not explicitly reflected in differences in Arc protein expression in either CA3 or CA1 subfields in the dorsal hippocampus

As reported above subjects trained in the three different fear conditioning tasks demonstrated contextual latent inhibition but freezing was not significantly different between different types of fear conditioning. While Arc protein in the dorsal hippocampus is critically necessary for learning context-US associations (Czerniawski et al., 2011), it is not differentially elevated following CFC compared to TFC or DFC where both CS-US and context-US associations are acquired. Arc protein was significantly elevated in dorsal CA3 for subjects who would later demonstrate contextually elicited fear suggesting that Arc expression within this region may reflect contextual learning which could later modulate differences in contextually-elicited fear. However, as discussed in Experiments 3 and 4, Arc

protein expression was not affected by context pre-exposure, which very clearly produced robust contextual latent inhibition, across different fear conditioning tasks, making it difficult to understand how a lack of differences in Arc enhancement could explicitly modulate differences in contextually-elicited fear.

Subjects trained in CFC were expected to demonstrate significantly greater enhancements in Arc protein in the dorsal hippocampus relative to subjects trained in TFC and DFC, however this was not observed. Actually, subjects trained TFC demonstrated significantly more Arc protein in dorsal CA3 compared to subjects trained in the other fear conditioning tasks.

Enhancement of Arc protein in dorsal CA3, but not CA1, is unique to subjects trained in trace fear conditioning suggesting an enhanced role for context-US representations

Arc protein expression was significantly enhanced in dorsal CA3 for subjects trained in TFC, not just above HC control subjects, but also compared to both CFC and DFC. Subjects trained in CFC and DFC both showed enhancement across CA1 and CA3 subfields, but only for TFC subjects was there a significant difference between subfields. This suggests that TFC specifically engages Arc dependent processes differently between dorsal CA3 and CA1. This is an intriguing difference as all of these fear conditioning tasks are sufficient to produce contextually-elicited fear, which would suggest a common role for the dorsal hippocampus across these fear conditioning task.

Why then is dorsal CA3 Arc protein expression particularly greater in TFC subjects? While both CA3 and CA1 are necessary for the expression of contextually elicited fear (Hunsaker & Kesner, 2008), the role of CA3 in TFC may be more related to learning the explicit CS-noUS contingency described above. Ultimately in TFC the CS is not paired with the US, but the trace interval following the CS. The CS provides a marker separating when the context precedes the US (trace interval) and when it does not (ITI). Hence, while during trace fear conditioning subjects are learning about the CS, this learning is mediated by the context. This interpretation is also supported by our own behavioral data in which context pre-exposure appears to affect conditioning responding to the CS for TFC but not DFC. Because the context is paired with the US in TFC, the CS can serve a different function of predicting noUS. Behavioral data reported above indicates that this kind of learning is facilitated by context familiarity suggesting CS and context learning to be integrally linked in TFC, while this is not the case for DFC subjects were CS learning is not affected by context pre-exposure.

Additionally, greater Arc protein enhancement in dorsal CA3 for subjects trained in TFC relative to CFC suggests the role of the context may be more complex. In CFC the context is directly paired with the US and no other stimuli are presented, while during TFC the context is paired with both the tone CS and the US. This splitting of the continuous context with the phasic CS may lead to a discrimination between the context prior to the CS, which is never present when the US occurs, from the context after the CS, which predicts the US (Kaplan & Hearst, 1982). This conditional discrimination of a context would require

additional contextual processing and differential spatial representation which would drive additional Arc protein expression in dorsal CA3 (Ramirez-Amaya et al., 2005; Vazdajanova and Guzowski, 2004; Guzowski 1999). Hence additional Arc protein enhancements in dorsal CA3 relative to other fear conditioning groups likely reflects the unique role the context plays in facilitating the CS-noUS contingency in TFC compared to DFC as well as the potential for conditional discrimination of the context following TFC (context predicting both the US and the CS) where in CFC the context only predicts the US.

Enhancement of Arc protein in ventral CA3, but not CA1, is unique to subjects trained in trace fear conditioning and may reflect explicit CS components of fear conditioning

Arc protein expression in ventral CA1 was significantly enhanced following both novel context exposure and all forms of fear conditioning (TFC, CFC, or DFC). Ventral CA1 provides the main path by which information in the hippocampus reaches the amygdala and while Arc protein expression in this region is very likely required to support fear conditioning, expression in this region is not unique to fear conditioning as enhancement is also seen after exposure to a novel context. Thus ventral CA1 may act as a relay station by which information from the hippocampus reaches the amygdala to facilitate learning about a potentially fearful and aversive context and does not reflect any specific components of the trace fear conditioning task (CS-US, context-US, or otherwise).

Ventral CA3 Arc protein expression, however, appears to be unique to subjects trained in TFC. While Arc protein was not significantly greater for subject trained in TFC compared to other groups, only for subjects trained in TFC was Arc protein significantly greater than HC controls. As discussed in detail in Experiment 3, ventral CA3 Arc protein expression may reflect processing the explicit tone CS in trace fear conditioning. Comparisons to other conditioning group supports this notion as Arc protein in CA3 is not significantly elevated in any other groups (Figure E5-4). For subjects trained in CFC there is no explicit CS to be represented by neuronal activity in CA3 which may account for the lack of Arc enhancement. For subjects trained in DFC interfering with ventral CA3 activity has no effect on CS learning (Hunsaker & Kesner, 2008) but does affect retrieval. For DFC this suggests that if the hippocampus is present during conditioning then it may be recruited in the processing of auditory-cued fear though it is not required for acquisition. Hence this same region may process this cue in trace fear conditioning. This suggest that activity in CA3 during trace fear conditioning may reflect auditory CS processing, hence preventing Arc protein expression in this region produces a deficit in trace fear conditioning. In regard to our account of a different functional role for the CS in TFC, blocking Arc protein enhancement in either the dorsal or ventral hippocampus impairs the acquisition of the CS-noUS contingency which in turn impairs the acquisition of both trace-US and context-US contingencies producing a global reduction in freezing (Czerniawski et al., 2011).

Conclusion

Across the dorsal and ventral hippocampus, Arc protein expression reflects different aspects of the experience, fear conditioning or otherwise. Within the dorsal hippocampus, Arc protein enhancements in both CA3 and CA1 reflect contextual components of the fear conditioning task. However, enhancement of Arc in dorsal CA3 following trace fear conditioning may play a unique role in representing the context. Here the context is represented with respect to its ability to predict of the presence or absence of the impending US. When comparing contextual representations between the three different types of conditioning in dorsal CA3, for TFC we may see two distinct populations of neurons, both sensitive to the context, with one population active prior to the CS, with a potentially different population of neurons after the CS.

Additionally, modulation of dorsal hippocampal activity and Arc protein expression by the CS may only be possible due to Arc protein expression in ventral CA3 reflecting the CS processing. Neurons in ventral CA3 may come to represent the explicitly trained CS to allow for a conditional discrimination of the context prior to the CS versus after the CS, as well as facilitate acquisition of the CS-noUS contingency. This additional hippocampal processing induced by the demands of the TFC task could account for greater Arc protein expression in dorsal and ventral CA3 for subjects trained in TFC compared to CFC and DFC subjects.

REFERENCES

Amaral D.G., & Lavenex P. (2007) Hippocampal neuroanatomy. In: Andersen P, Morris R, Amaral D, Bliss T, O'Keefe J, editors. *The Hippocampus Book*. Oxford University Press; New York: p. 872.

Anagnostaras, S. G., Maren, S., & Fanselow, M. S. (1999). Temporally graded retrograde amnesia of contextual fear after hippocampal damage in rats: within-subjects examination. *The Journal of neuroscience*, *19*(3), 1106-1114.

Andersen, P. (Ed.). (2007). *The hippocampus book*. Oxford university press.

Barot, S. K., Chung, A., Kim, J. J., & Bernstein, I. L. (2009). Functional imaging of stimulus convergence in amygdalar neurons during Pavlovian fear conditioning. *PLoS One, 4*(7), e6156.

Bauer, E. P., Schafe, G. E., & LeDoux, J. E. (2002). NMDA receptors and L-type voltage-gated calcium channels contribute to long-term potentiation and different components of fear memory formation in the lateral amygdala. *The Journal of neuroscience, 22*(12), 5239-5249.

Beer, Z., Chwiesko, C., Kitsukawa, T., & Sauvage, M. M. (2013). Spatial and stimulus-type tuning in the LEC, MEC, POR, PrC, CA1, and CA3 during spontaneous item recognition memory. *Hippocampus*, *23*(12), 1425-1438.

Beer, Z., Chwiesko, C., & Sauvage, M. M. (2014). Processing of spatial and non-spatial information reveals functional homogeneity along the dorso-ventral axis of CA3, but not CA1. *Neurobiology of learning and memory, 111*, 56-64.

Bliss, T. V., & Lomo, T. (1973). Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. *The Journal of physiology, 232*(2), 331-356.

Bliss, T. V., & Collingridge, G. L. (1993). A synaptic model of memory: long-term potentiation in the hippocampus. *Nature, 361*(6407), 31-39.

Bliss, T., Collingridge, G., & Morris, R. G. M. (2007). The hippocampus book. Andersen, P, 343-474.

Bolles, R. C., Collier, A. C., Bouton, M. E., & Marlin, N. A. (1978). Some tricks for ameliorating the trace-conditioning deficit. *Bulletin of the Psychonomic Society, 11*(6), 403-406.Boughner et al., 2004

Bramham C.R., Worley P.F., Moore M.J., & Guzowski J.F. (2008) The immediate early gene arc/arg3.1: Regulation, mechanisms, and function. *Journal of Neuroscience*, *28*, 11760-11767.

Burns L.H., Annett L., Kelley A.E., Everitt B.J., & Robbins T.W. (1996) Effects of lesions to amygdala, ventral subiculum, medial prefrontal cortex, and nucleus accumbens on the reaction to novelty: Implication for limbic-striatal interactions. *Behavioral Neuroscience, 110*:60–73.

Canteras, N. S., & Swanson, L. W. (1992). Projections of the ventral subiculum to the amygdala, septum, and hypothalamus: A PHAL anterograde tract-tracing study in the rat. *Journal of Comparative Neurology*, *324*(2), 180-194.

Chia C., & Otto T., (2013) Hippocampal Arc (Arg3.1) expression is induced by memory recall and required for memory reconsolidation in trace fear conditioning. *Neurobiology of Learning and Memory*, *106*:48-55.

Cohen, N. J., & Squire, L. R. (1980). Preserved learning and retention of pattern-analyzing skill in amnesia: Dissociation of knowing how and knowing that. *Science, 210*(4466), 207-210.

Cole, R. P., Barnet, R. C., & Miller, R. R. (1995). Temporal encoding in trace conditioning. *Animal Learning & Behavior, 23*(2), 144-153.Corcoran & Maren, 2001

Czerniawski J., Ree F., Chia C., & Otto T. (2012) Dorsal vs. ventral hippocampal contributions to trace and contextual conditioning: Differential effects of regionally selective NMDA receptor antagonism on acquisition and expression. *Hippocampus*, *22,* 1528-1539.

Czerniawski J., Yoon T., Otto T. (2009) Dissociating space and trace in dorsal and ventral hippocampus. *Hippocampus*, *19*:20 –32.

Czerniawski J., Ree F., Chia C., Ramamoorthi K., Kumata Y., & Otto T.A. (2011) The importance of having Arc: expression of the immediate-early gene Arc is required for hippocampus dependent fear conditioning and blocked by NMDA receptor antagonism. *Journal of Neuroscience, 31*(31), 11200-11207.

Davitz, J. R., Mason, D. J., Mowrer, O. H., & Viek, P. (1957). Conditioning of fear: A function of the delay of reinforcement. *The American journal of psychology, 70*(1), 69-74.

Dere, E., Huston, J. P., & Silva, M. A. D. S. (2005). Integrated memory for objects, places, and temporal order: evidence for episodic-like memory in mice. *Neurobiology of learning and memory, 84*(3), 214-221.

Dunn L.T., & Everitt B.J. (1988) Double dissociations of the effects of amygdala and insular cortex lesions on conditioned taste aversion, passive avoidance, and neophobia in the rat using excitotoxin ibotenic acid. *Behavioral Neuroscience*, *102*:3–23.

Eichenbaum H. (1996) Is the rodent hippocampus just for ‘‘place’’? *Current Opinions in Neurobiology, 6*:187–195.

Eichenbaum, H., Schoenbaum, G., Young, B., & Bunsey, M. (1996). Functional organization of the hippocampal memory system. *Proceedings of the National Academy of Sciences, 93(*24), 13500-13507.

Eichenbaum, H., Dudchenko, P., Wood, E., Shapiro, M., & Tanila, H. (1999). The hippocampus, memory, and place cells: is it spatial memory or a memory space?. *Neuron, 23*(2), 209-226.

Eichenbaum, H., Komorowski, R., MacDonald, C. J., Kraus, B. J., & Robitsek, J. (2016). How Does the Hippocampus Support the Spatial and Temporal

Attributes of Memory?. *In The Neurobiological Basis of Memory* (pp. 39-57). Springer International Publishing.

Escobar, M., Arcediano, F., & Miller, R. R. (2002). Latent inhibition and contextual associations. Journal of Experimental Psychology: *Animal Behavior Processes, 28*(2), 123.

Fanselow, M. S. (1980). Conditional and unconditional components of post-shock freezing. *The Pavlovian journal of biological science: Official Journal of the Pavlovian, 15*(4), 177-182.

Fanselow, M. S. (1986). Associative vs topographical accounts of the immediate shock-freezing deficit in rats: implications for the response selection rules governing species-specific defensive reactions. *Learning and Motivation, 17*(1), 16-39.

Fanselow, M. S. (1990). Factors governing one-trial contextual conditioning. *Animal Learning & Behavior, 18*(3), 264-270.

Fanselow, M. S., & Poulos, A. M. (2005). The neuroscience of mammalian associative learning. *Annu. Rev. Psychol., 56*, 207-234.

Fanselow M.S., Dong H. (2010) Are the dorsal and ventral hippocampus functionally distinct structures? *Neuron, 65*, 7–19.

Fletcher B.R., Calhoun M.E., Rapp P.R., Shapiro M.L. (2006) Fornix lesions decouple the induction of hippocampal Arc transcription from behavior but not plasticity. *Journal of Neuroscience, 26*:1507–1515.

Frankland, P. W., Cestari, V., Filipkowski, R. K., McDonald, R. J., & Silva, A. J. (1998). The dorsal hippocampus is essential for context discrimination but not for contextual conditioning. *Behavioral neuroscience, 112*(4), 863.

Frankland, P. W., Ding, H. K., Takahashi, E., Suzuki, A., Kida, S., & Silva, A. J. (2006). Stability of recent and remote contextual fear memory. *Learning & Memory, 13*(4), 451-457.

Gallistel, C. R., & Gibbon, J. (2000). Time, rate, and conditioning. *Psychological review, 107*(2), 289.

Gilbert P.E., & Kesner R.P. (2004) Memory for objects and their locations: The role of the hippocampus in retention of object–place associations, *Neurobiology of Learning and Memory, 81*(1):39-45.

Goodrich-Hunsaker N. J., Hunsaker, M.R., & Kesner, R.P. (2008) The interactions and dissociations of the dorsal hippocampus subregions: How the dentate gyrus, CA3, and CA1 process spatial information. *Behavioral Neuroscience, 122*(1):16-26.

Guzowski J.F., McNaughton B.L., Barnes C.A., Worley P.F. (1999) Environment-specific expression of the immediate-early gene Arc in hippocampal neuronal ensembles. *Nature Neuroscience, 2*:1120 –1124.

Guzowski J.F., Lyford G.L., Stevenson G.D., Houston F.P., McGaugh J.L., Worley P.F., Barnes C.A. (2000) Inhibition of activity-dependent Arc protein expression in the rat hippocampus impairs the maintenance of long-term

potentiation and consolidation of long-term memory. *Journal of Neuroscience, 20*: 3993–4001.

Guzowski J.F. (2002) Insights into immediate-early gene function in hippocampal memory consolidation using antisense oligonucleotide and fluorescent imaging approaches. *Hippocampus, 12*(1) 86-104.

Guzowski, J. F., Knierim, J. J., & Moser, E. I. (2004). Ensemble dynamics of hippocampal regions CA3 and CA1. *Neuron, 44*(4), 581-584.

Hebb, D. O. (1949). *The organization of behavior: A neuropsychological approach*. John Wiley & Sons.

Herring, B. E., & Nicoll, R. A. (2016). Long-term potentiation: from CaMKII to AMPA receptor trafficking. *Annual review of physiology, 78*, 351-365.

Hoang L.T., & Kesner R.P. (2008) Dorsal hippocampus, CA3 and CA1 lesions disrupt temporal sequence completion. *Behavioral Neuroscience*, 122(1): 9-15.

Hoge J., & Kesner R.P. (2007) Role of CA3 and CA1 subregions of the dorsal hippocampus on temporal processing of objects. *Neurobiology of Learning and Memory*, 88(2): 225-231.

Hunsaker, M. R., & Kesner, R. P. (2008). Dissociations across the dorsal–ventral axis of CA3 and CA1 for encoding and retrieval of contextual and auditory-cued fear. *Neurobiology of learning and memory, 89*(1), 61-69.

Hunsaker, M. R., Fieldsted, P. M., Rosenberg, J. S., & Kesner, R. P. (2008) Dissociating the roles of dorsal and ventral CA1 for the temporal processing of spatial locations, visual objects, and odors. *Behavioral Neuroscience*, 122, 643–650.

Hunsaker M.R., Thorup J.A., Welch T., & Kesner R.P. (2006) The role of CA3 and CA1 in the acquisition of an object–trace–place paired-associate task. *Behavioral Neuroscience*, 120(6):1252-1256.

Izquierdo, I., Furini, C. R., & Myskiw, J. C. (2016). Fear memory. *Physiological reviews, 96(*2), 695-750.

Jung, M. W., Wiener, S. I., & McNaughton, B. L. (1994). Comparison of spatial firing characteristics of units in dorsal and ventral hippocampus of the rat. *The Journal of neuroscience, 14*(12), 7347-7356.

Kamin, L. J. (1954). Traumatic avoidance learning: the effects of CS-US interval with a trace-conditioning procedure. *Journal of Comparative and Physiological Psychology, 47*(1), 65.

Kamin, L. J. (1961). Apparent adaptation effects in the acquisition of a conditioned emotional response. *Canadian Journal of Psychology/Revue canadienne de psychologie, 15*(3), 176.

Kaplan, P. S., & Hearst, E. (1982). Bridging temporal gaps between CS and US in autoshaping: insertion of other stimuli before, during, and after CS. *Journal of Experimental Psychology: Animal Behavior Processes, 8*(2), 187.

Kaplan, P. S. (1984). Importance of relative temporal parameters in trace autoshaping: From excitation to inhibition. *Journal of Experimental Psychology: Animal Behavior Processes, 10*(2), 113.

Kesner R.P. (2007) Behavioral functions of the CA3 subregion of the hippocampus. *Learning and Memory*,14: 771-781.

Kesner R.P., Hunsaker M.R., & Ziegler W. (2010) The role of the dorsal CA1 and ventral CA1 in memory for the temporal order of a sequence of odors. *Neurobiology of Learning and Memory*, 93, 111-116.

Kim, J. J., & Jung, M. W. (2006). Neural circuits and mechanisms involved in Pavlovian fear conditioning: a critical review. *Neuroscience & Biobehavioral Reviews, 30*(2), 188-202.

Kim, J. J., & Fanselow, M. S. (1992). Modality-specific retrograde amnesia of fear. *Science, 256*(5057), 675.

Knierim, J. J., Kudrimoti, H. S., & McNaughton, B. L. (1995). Place cells, head direction cells, and the learning of landmark stability. *The Journal of Neuroscience, 15*(3), 1648-1659.

Korb, E., & Finkbeiner, S. (2011). Arc in synaptic plasticity: from gene to behavior. *Trends in neurosciences, 34*(11), 591-598.

Lanahan, A., & Worley, P. (1998). Immediate-early genes and synaptic function. *Neurobiology of learning and memory, 70*(1), 37-43.

LeDoux, J. E. (1995). Emotion: Clues from the brain. *Annual review of psychology, 46*, 209.

LeDoux, J. (2000). Emotion circuits in the brain, *Annual Review of Neuroscience, 23*, pp. 155-84.

Lee, I., Jerman, T.S., & Kesner, R.P. (2005). Disruption of delayed memory for a sequence of spatial locations following CA1- or CA3-lesions of the dorsal hippocampus. *Neurobiology of Learning and Memory, 84,* 138–147.

Li M., Hou Y., Lu B., Chen J., Chi Z., & Liu J. (2009) Expression pattern of neural synaptic plasticity marker-Arc in different brain regions induced by conditioned drug withdrawal from acute morphine-dependent rats. *Acta Pharmacol Sin*, 30(3):282-290.

Link W., Konietzko U., Kauselmann G., Krug M., Schwanke B., Frey U., & Kuhl D. (1995) Somatodendritic expression of an immediate early gene is regulated by synaptic activity. *Proceedings of the National Academy of Sciences U S A*, 92:5734 –5738.

Lomo, T. (1966, January). Frequency potentiation of excitatory synaptic activity in dentate area of hippocampal formation. *In Acta Physiologica Scandinavica* (p. 128).

Lonergan, M. E., Gafford, G. M., Jarome, T. J., & Helmstetter, F. J. (2010). Time-dependent expression of Arc and zif268 after acquisition of fear conditioning. *Neural plasticity, 2010*.

Lyford G.L., Yamagata K., Kaufmann W.E., Barnes C.A., Sanders L.K., Copeland N.G., Gilbert D.J., Jenkins N.A., Lanahan A.A., & Worley P.F. (1995) Arc, a growth factor and activity regulated gene, encodes a novel cytoskeleton-associated protein that is enriched in neuronal dendrites. *Neuron*, 14:433–445.

Maren, S., Aharonov, G., & Fanselow, M. S. (1997). Neurotoxic lesions of the dorsal hippocampus and Pavlovian fear conditioning in rats. *Behavioural brain research, 88(*2), 261-274.

Maren, S. (2001). Neurobiology of Pavlovian fear conditioning. *Annual review of neuroscience, 24*(1), 897-931.

Maren, S., & Holt, W. G. (2004). Hippocampus and Pavlovian fear conditioning in rats: Muscimol infusions into the ventral, but not dorsal, hippocampus impair the acquisition of conditional freezing to an auditory conditional stimulus. *Behavioral Neuroscience*, 118, 97–110.

Matzel, L. D., Held, F. P., & Miller, R. R. (1988). Information and expression of simultaneous and backward associations: Implications for contiguity theory. *Learning and motivation, 19(*4), 317-344.

McIntyre C.K., Miyashita T., Setlow B., Marjon K.D., Steward O., Guzowski J.F., & McGaugh J.L. (2005) Memory-influencing intra-basolateral amygdala drug infusions modulate expression of Arc protein in the hippocampus. *Proceedings of the National Academy of Sciences USA*, 102:10718 –10723.

McNaughton B.L., & Morris R.G.M. (1987) Hippocampal synaptic enhancement and information storage within a distributed memory system. *Trends in Neurosciences*, 10(10), 408-415.

Milner, B., Corkin, S., & Teuber, H. L. (1968). Further analysis of the hippocampal amnesic syndrome: 14-year follow-up study of HM. *Neuropsychologia, 6(*3), 215-234.

Misane, I., Tovote, P., Meyer, M., Spiess, J., Ögren, S.O. and Stiedl, O., (2005). Time-dependent involvement of the dorsal hippocampus in trace fear conditioning in mice. *Hippocampus, 15*(4), pp.418-426.

Monti M.C., Almiron R.S., Bignante E.A., & Ramirez O.A. (2010). Changes in hippocampal Arc protein expression and synaptic plasticity by the presentation of contextual cues liked to drug experience. *Synapse*, 64:39-46.

Moser M.B., & Moser E.I. (1998). Functional differentiation in the hippocampus. *Hippocampus*, 8:608–619.

Moses S.N., Sutherland R.J., & McDonald R.J. (2002). Differential involvement of amygdala and hippocampus in responding to novel objects and contexts. *Brain Research Bulletin*, 58(5), 517-527.

Mowrer, O. H., & Lamoreaux, R. R. (1951). Conditioning and conditionality (discrimination). *P`sychological Review, 58*(3), 196.

O'Keefe, J., & Dostrovsky, J. (1971). The hippocampus as a spatial map. Preliminary evidence from unit activity in the freely-moving rat. *Brain research, 34*(1), 171-175.

O'Keefe, J., & Nadel, L. (1978).*The hippocampus as a cognitive map*. Oxford:Clarendon Press.

Orsini, C. A., & Maren, S. (2012). Neural and cellular mechanisms of fear and extinction memory formation. *Neuroscience & Biobehavioral Reviews, 36*(7), 1773-1802.

Parsons, T. C., & Otto, T. (2008). Temporary inactivation of dorsal hippocampus attenuates explicitly nonspatial, unimodal, contextual fear conditioning. *Neurobiology of learning and memory, 90*(1), 261-268.

Parsons, T. C., & Otto, T. (2010). Time-limited involvement of dorsal hippocampus in unimodal discriminative contextual conditioning. *Neurobiology of learning and memory, 94(*4), 481-487.

Pavlov, I. P. (1927). Conditioned reflexes. *An Investigation of the physiological activity of the cerebral cortex.*

Paxinos G., & Watson C. (2007). The rat brain in stereotaxic coordinates. Boston: Elsevier Academic.

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

Ploski J.E., Pierre V.J., Smucny J., Park K., Monsey M.S., Overeem K.A., & Schafe, G.E. (2008). The activity-regulated cytoskeletal-associated protein (Arc/ Arg3.1) is required for memory consolidation of Pavlovian fear conditioning in the lateral amygdala. *Journal of Neuroscience,* 28:12383–12395.

Pitkanen A., Pikkarainen M., Nurminen N., & Ylinen A. (2000). Reciprocal connections between the amygdala and hippocampal formation, perirhinal cortex, and postrhinal cortex in rat. *Annual NY Academy of Science*, 911:369–391.

Pyapali G.K., Sik A., Penttonen M., Buzsaki G., & Turner D.A. (1998). Dendrite properties of hippocampal CA1 pyramidal neurons in the rat: intracellular staining in vivo and in vitro. *The Journal of Comparative Neurology*, 391, 335-352.

Ramamoorthi, K., Fropf, R., Belfort, G. M., Fitzmaurice, H. L., McKinney, R. M., Neve, R. L., Otto, T., & Lin, Y. (2011). Npas4 regulates a transcriptional program in CA3 required for contextual memory formation. *Science, 334*(6063), 1669-1675.

Ramirez-Amaya V., Vazdarjanova A., Mikhael D., Rosi S., Worley P.F., & Barnes C.A. (2005). Spatial exploration-induced Arc mRNA and protein expression: evidence for selective, network-specific reactivation. *Journal of Neuroscience*, 25(7), 1761-1768.

Ramirez, S., Liu, X., Lin, P. A., Suh, J., Pignatelli, M., Redondo, R. L., ... & Tonegawa, S. (2013). Creating a false memory in the hippocampus. *Science, 341*(6144), 387-391.

Rescorla, R. A., & Wagner, A. R. (1972). A theory of Pavlovian conditioning: Variations in the effectiveness of reinforcement and nonreinforcement. Classical conditioning II: *Current research and theory, 2*, 64-99.

Risold P.Y., & Swanson L.W. (1996) Structural evidence for functional domains in the rat hippocampus. *Science*, 272:1484-1486.

Rogers J.L., Hunsaker M.R., & Kesner R.P. (2006) Effects of dorsal and ventral CA1 subregional lesions on trace fear conditioning. *Neurobiology of Learning and Memory*, 8, 72–81.

Rolls E.T., & Kesner R.P. (2006) A computational theory of hippocampal function, and empirical tests of the theory. *Progress in Neurobiology*, 79:1-48

Rolls E.T., & Rolls B.J. (1973) Altered food preferences after lesions in the basolateral region of the amygdala in the rat. *Journal of Comparative Physiology & Psychology*, 83:248–259.

Rudy, J. W., & Matus-Amat, P. (2005). The ventral hippocampus supports a memory representation of context and contextual fear conditioning: Implications for a unitary function of the hippocampus. Behavioral Neuroscience, 119, 154–163.

Scoville, W. B., & Milner, B. (1957). Loss of recent memory after bilateral hippocampal lesions. *Journal of Neurology, Neurosurgery & Psychiatry, 20*(1), 11-21.

Shepherd, J. D., Rumbaugh, G., Wu, J., Chowdhury, S., Plath, N., Kuhl, D., ... & Worley, P. F. (2006). Arc/Arg3. 1 mediates homeostatic synaptic scaling of AMPA receptors. *Neuron, 52*(3), 475-484.

Shepherd, J. D., & Bear, M. F. (2011). New views of Arc, a master regulator of synaptic plasticity. *Nature neuroscience, 14*(3), 279-284.

Shohamy, D., Allen, M. T., & Gluck, M. A. (2000). Dissociating entorhinal and hippocampal involvement in latent inhibition. *Behavioral Neuroscience, 114*(5), 867.

Squire, L. R., Stark, C. E., & Clark, R. E. (2004). The medial temporal lobe*. *Annu. Rev. Neurosci., 27*, 279-306.

Steward O., Wallace C.S., Lyford G.L., & Worley P.F. (1998) Synaptic activation causes the mRNA for the IEG Arc to localize selectively near activated postsynaptic sites on dendrites. *Neuron*, 21:741–751.

Sutherland R.J., & McDonald R.J. (1990) Hippocampus, amygdala, and memory deficits in rats. *Behavioral Brain Research*, 37:57–59.

Sutherland, R. J., Sparks, F. T., & Lehmann, H. (2010). Hippocampus and retrograde amnesia in the rat model: a modest proposal for the situation of systems consolidation. *Neuropsychologia, 48*(8), 2357-2369.

Sutherland, R. J., & Lehmann, H. (2011). Alternative conceptions of memory consolidation and the role of the hippocampus at the systems level in rodents. *Current opinion in neurobiology, 21*(3), 446-451.

Tanaka, K. Z., Pevzner, A., Hamidi, A. B., Nakazawa, Y., Graham, J., & Wiltgen, B. J. (2014). Cortical representations are reinstated by the hippocampus during memory retrieval. *Neuron, 84*(2), 347-354.

Thrailkill, E. A., & Shahan, T. A. (2014). Temporal integration and instrumental conditioned reinforcement. *Learning & behavior, 42*(3), 201-208.

Vago D.R., Bevan A., & Kesner R.P. (2007) The role of the direct perforant path input to the CA1 subregion of the dorsal hippocampus in memory retention and retrieval. *Hippocampus*, 17(10), 977-987.

Vazdarjanova A. & Guzowski J.F. (2004). Differences in hippocampal neuronal population responses to modifications. *The Journal of Neuroscience*, 24(29), 6489-6496.

Vazdarjanova, A., Ramirez-Amaya, V., Insel, N., Plummer, T. K., Rosi, S., Chowdhury, S., ... & Barnes, C. A. (2006). Spatial exploration induces ARC, a plasticity-related immediate-early gene, only in calcium/calmodulin-dependent protein kinase II-positive principal excitatory and inhibitory neurons of the rat forebrain. *Journal of Comparative Neurology, 498*(3), 317-329.

Verde, E. M. R., Lee-Osbourne, J., Worley, P. F., Malinow, R., & Cline, H. T. (2006). Increased expression of the immediate-early gene arc/arg3.1 reduces AMPA receptor-mediated synaptic transmission. *Neuron, 52*(3), 461-474.

Waung, M. W., Pfeiffer, B. E., Nosyreva, E. D., Ronesi, J. A., & Huber, K. M. (2008). Rapid translation of Arc/Arg3.1 selectively mediates mGluR-dependent LTD through persistent increases in AMPAR endocytosis rate. *Neuron, 59*(1), 84-97.

Yoon T., & Otto T. (2007). Differential contributions of the dorsal and ventral hippocampus in rats to trace fear conditioning. *Neurobiology of Learning and Memory, 87*:464–475.

Zhang, S., & Manahan-Vaughan, D. (2015). Spatial olfactory learning contributes to place field formation in the hippocampus. *Cerebral Cortex, 25*(2), 423-432.