Chronic stress and plasticity in the limbic system:

Implications for post traumatic stress disorder

by

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ABSTRACT

The brain is a fundamental target of the stress response that promotes adaptation and

survival but the repeated activation of the stress response has the potential alter cognition,

emotion, and motivation, key functions of the limbic system. Three structures of the

limbic system in particular, the hippocampus, medial prefrontal cortex (mPFC), and

amygdala, are of special interest due to documented structural changes and their

implication in post-traumatic stress disorder (PTSD). One of many notable chronic stress-

induced changes include dendritic arbor restructuring, which reflect plasticity patterns in

parallel with the direction of alterations observed in functional imaging studies in PTSD

patients. For instance, chronic stress produces dendritic retraction in the hippocampus

and mPFC, but dendritic hypertrophy in the amygdala, consistent with functional imaging

in patients with PTSD. Some have hypothesized that these limbic region's modifications

contribute to one's susceptibility to develop PTSD following a traumatic event.

Consequently, we used a familiar chronic stress procedure in a rat model to create a

vulnerable brain that might develop traits consistent with PTSD when presented with a

challenge. In adult male rats, chronic stress by wire mesh restraint (6h/d/21d) was

followed by a variety of behavioral tasks including radial arm water maze (RAWM), fear

conditioning and extinction, and fear memory reconsolidation to determine chronic stress

effects on behaviors mediated by these limbic structures. In chapter 2, we corroborated

past findings that chronic stress caused hippocampal CA3 dendritic retraction.

Importantly, we present new findings that CA3 dendritic retraction corresponded with

poor spatial memory in the RAWM and that these outcomes reversed after a recovery

period. In chapter 3, we also showed that chronic stress impaired mPFC-mediated

extinction memory, findings that others have reported. Using carefully assessed behavior, we present new findings that chronic stress impacted nonassociative fear by enhancing contextual fear during extinction that generalized to a new context. Moreover, the generalization behavior corresponded with enhanced functional activation in the hippocampus and amygdala during fear extinction memory retrieval. In chapter 5, we showed for the first time that chronic stress enhanced amygdala functional activation during fear memory retrieval, i.e., reactivation. Moreover, these enhanced fear memories were resistant to protein synthesis interference to disrupt a previously formed memory, called reconsolidation in a novel attempt to weaken chronic stress enhanced traumatic memory. Collectively, these studies demonstrated the plastic and dynamic effects of chronic stress on limbic neurocircuitry implicated in PTSD. We showed that chronic stress created a structural and functional imbalance across the hippocampus, mPFC, and amygdala, which lead to a PTSD-like phenotype with persistent and exaggerated fear following fear conditioning. These behavioral disruptions in conjunction with morphological and functional imaging data reflect a chronic stress-induced imbalance between hippocampal and mPFC regulation in favor of amygdala function overdrive, and supports a novel approach for traumatic memory processing in PTSD.

To my parents, Rose and Kip Hoffman, for always offering me your unconditional love

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CHAPTER 1

GENERAL INTRODUCTION

Stress is a multidimensional construct that is typically used to define an

organism's response to a threat. The stress response is highly conserved to indicate that

the stress process successfully guides survival in many organisms. For example, soldiers

exposed to combat stress are often confronted by true and anticipated threats to survival.

For these events, the sympathetic nervous system (SNS) releases epinephrine and

norepinephrine and launches the "fight or flight" response to redirect resources where it

is needed, producing effects such as dilated pupils, dilated blood vessels in skeletal

muscle, and increased his heart rate. These rapid effects allow the soldier to maintain

vigilance and react quickly. Meanwhile, the hypothalamic-pituitary-adrenal (HPA) axis

becomes engaged, a cascade of events known as the neuroendocrine stress response.

Upon detection of a stressor, the paraventricular nucleus of the hypothalamus (PVN)

stimulates the release of corticotropin releasing hormone (CRH), which stimulates the

release of adrenocorticotropic hormone (ACTH) from the anterior pituitary into the

systemic circulation. ACTH elicits the release of glucocorticoids (GCs) from the adrenal

cortex into the blood to reach peripheral and central target tissues. As the threat recedes,

circulating GCs reach the pituitary and areas of the brain (PVN and hippocampus) to halt

the production of these stress hormones, an important regulatory step in the maintenance

of an adapting system. This example illustrates that stress is an essential process that

promotes adaptation and survival.

Conventional definitions of stressors have evolved over time to be a real or

perceived threat to homeostasis, or an anticipated threat to ego or well-being, and can be

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categorized by the response mechanism (Herman, 2011). For example, hemorrhage, a real homeostatic stressor due to a gunshot wound in combat, would result in direct activation of the SNS and HPA axis. However, anticipated threats make up a large proportion of stressors that vary across individuals, and can range from the expectation of enemy approach as in our soldier example to day to day stressors in modern society such as meeting deadlines, paying bills, and difficulties in interpersonal relationships. Moreover, one individual's anticipation of threat may not be perceived similarly by another, which adds to individual variability. Generally, these types of perceived threats depend on interpretation of sensory stimuli with respect to previous experience (Herman *et al.*, 2003; Ulrich-Lai & Herman, 2009), and indirectly stimulate the SNS and HPA axis (Herman, 2011). Regardless of the stressor type, the acute stress response can be critical to survival, but how our systems adapt to multiple or repeated stressors over time might lead to detrimental outcomes.

Repeated activation of the stress response, or what is often referred to as chronic stress, can lead to wear and tear on the body and brain, and ultimately lead to pathophysiology and/or susceptibility to psychiatric conditions (McEwen, 2007). Allostasis is a term that was introduced to describe an additional process of reestablishing homeostasis through active processes in response to experiences, such as stress. These active process can lead to changes in receptor expression, synapse formation or pruning and altered neuronal dendritic morphology and neurogenesis. Chronic stress is a process that gives rise to allostasis, and as a consequence, this adapting set point can eventually lead to allostatic load or overload, and result in insufficient adaptation to additional stress (McEwen, 2004). For example, a downregulation of a particular receptor might mean that

the organism is less sensitive to the type of neurotransmitter or hormone that would bind

to it and consequently, the chronically stressed organism might require a greater stimulus

to achieve the same response prior to chronic stress. These consequences can manifest as

a weakened immune response, hypertension, obesity, and neuronal atrophy within the

central nervous system (McEwen, 2004).

An important regulator of allostasis is the brain, as it determines whether a

stimulus is to be avoided or approached, and controls the subsequent behavioral and

physiological responses (McEwen, 2007). Within the brain and a target of the stress

response is the limbic system, a complex collection of brain structures that support a

variety of functions including emotion, motivation, learning and memory. In this

dissertation, a subset of limbic structures that includes the hippocampus, medial

prefrontal cortex (mPFC) and amygdala are the focus because they are intimately

involved in stress, cognition, and emotion. Moreover, the plastic nature of these

structures and subsequent structural and functional dynamic alterations in response to

chronic stress are hypothesized to play a role in the susceptibility to develop psychiatric

conditions (McEwen, 2004), such as post traumatic stress disorder (PTSD), which is the

focus of the current research.

In the class of trauma and stressor-related disorders, PTSD is a debilitating and

complex anxiety disorder that sometimes manifests following a traumatic event

(American Psychiatric Association, 2013). PTSD symptoms include: (1) intrusive re-

experiencing of the event in the form of nightmares and flashbacks, with an exaggerated

response to trauma-related reminders and cues; (2) avoidance of stimuli associated with

the trauma and emotional numbing; (3) negative alterations in cognition and mood; and

(4) symptoms of an exaggerated startle response, increased physiological arousal, and sustained preparedness for an instant alarm response (American Psychiatric Association, 2013). Moreover, symptoms must persist for at least one month or more following the trauma, (American Psychiatric Association, 2013). One study reported that approximately 20% to 30% of individuals exposed to traumatic stressors will develop PTSD (Breslau *et al.*, 1991), with a lifetime prevalence of about 7% in the general population (Fairbank *et al.*, 1995; Kessler *et al.*, 2005). We still do not have a clear biological basis for understanding the full complexity of PTSD and why individuals respond differently to seemingly similar stressful episodes. The discordance between populations of people exposed to a traumatic event that develop PTSD and those that do not, suggest differences in vulnerability and/or resilience and emphasizes the need for more studies to determine etiology.

To investigate brain areas impacted in patient populations with PTSD, functional neuroimaging studies in clinical cases reveal some consistent differences in the PTSD brain compared to healthy controls. Specifically, PTSD patients show structural and functional alterations in limbic structures that are targets of the stress response, including the hippocampus, mPFC, and amygdala. One review of the clinical imaging literature in PTSD populations suggests a predominant finding of decreased hippocampal volume that is inversely associated with symptom severity (Shin *et al.*, 2006). Moreover, magnetic resonance spectroscopy studies report decreased hippocampal N-acetylaspartate (NAA) in PTSD, a measure of neuronal integrity (Schuff *et al.*, 2001; Brown *et al.*, 2003). Similar to the hippocampus, imaging studies have reported decreased mPFC volumes (Rauch *et al.*, 2003; Woodward *et al.*, 2006), and decreased NAA levels in PTSD (De

Bellis *et al.*, 2000), suggesting decreased neuronal integrity in the mPFC. Furthermore,

functional imaging studies in PTSD consistently report decreased activation and/or

activation failure in the mPFC to trauma related stimuli (Shin *et al.*, 2004; Shin *et al.*,

2006), which suggests difficulties in emotional regulation. In contrast to the hippocampus

and mPFC, functional neuroimaging studies support amygdala hyperresponsivity to both

traumatic reminders and general affective stimuli, and a positive correlation between

PTSD symptom severity and amygdala activation (Shin *et al.*, 2006). Although a first

step in understanding the neurobiology of PTSD is identifying differences in brain

function among those with and without the disorder, it is unknown whether these

alterations are contributing factors to, or outcomes from the disorder or trauma exposure.

The vast majority of people that experience traumatic stress do not develop PTSD

(Breslau *et al.*, 1991); and while the nature of each trauma is heterogeneous and

unpredictable, individuals that develop PTSD following trauma exposure may have

similar underlying vulnerabilities that warrant attention. Clinical evidence suggests that a

history of prior trauma is associated with increased risk for PTSD following subsequent

trauma (Davidson *et al.*, 1991; Bremner *et al.*, 1993; Zaidi & Foy, 1994; Breslau *et al.*,

1999). A recent study reported that 72.1% of ex-prisoners of war displayed PTSD, while

88.8% of control veterans were classified as "resilient," having no symptoms of PTSD

(Solomon *et al.*, 2012). One hypothesis suggests that a vulnerable brain increases the

susceptibility for the development of PTSD, which could stem from either environmental

or genetic factors. The latter has been supported by clinical evidence in twin studies

(Gilbertson *et al.*, 2002; Gurvits *et al.*, 2006). Specifically, in monozygotic twins

discordant for combat exposure, Gilbertson et al. (2002) revealed a significant negative

correlation between PTSD symptom severity in the PTSD combat exposed twin and hippocampal volume in the unexposed twin. In other words, smaller hippocampi in PTSD demonstrated a pre-existing, familial vulnerability factor rather than a product of trauma exposure in itself. More studies are needed to identify risk factors and individual differences for the development of PTSD, while animal models can help approach the questions raised in clinical research in prospective designs under controllable conditions.

The hippocampus, mPFC, and amygdala limbic circuit is critical in processing emotional memories and is disrupted in PTSD. Understanding the functional circuitry under healthy conditions is a first step in uncovering the neural basis of pathological fear memories. The amygdala is well understood to be the center for processing emotional stimuli is situated in the anteromedial temporal lobe, ventral and slightly anterior to the dorsal hippocampus. The amygdala is connected to a wide range of sensory and cognitive brain structures including the hippocampus and mPFC (Romanski & LeDoux, 1993; LeDoux, 2000; 2003; Hartley & Phelps, 2010). The amygdala has reciprocal excitatory projections with the hippocampus (Pitkanen *et al.*, 2000). Amygdala-hippocampal projections are known to play a role in hippocampal processing in response to stress and emotional memory formation (Akirav & Richter-Levin, 1999; McGaugh, 2004), whereas hippocampal projections to the amygdala and mPFC have been shown to modulate context-dependent fear extinction and emotional regulation (Maren & Quirk, 2004; Quirk & Mueller, 2008; Hartley & Phelps, 2010; Sotres-Bayon & Quirk, 2010). The mPFC has reciprocal excitatory connections with the amygdala (Maren & Quirk, 2004; Quirk *et al.*, 2006; Quirk & Mueller, 2008; Sotres-Bayon & Quirk, 2010). While the mPFC-amygdala network is understood to modulate amygdala output and fear inhibition following

extinction (Milad & Quirk, 2002; Quirk *et al.*, 2003; Quirk & Mueller, 2008), amygdala-mPFC projections play a role in the expression of learned fear (Sotres-Bayon & Quirk, 2010). Though the hippocampus directly projects to the mPFC, there are no direct projections from mPFC to the hippocampus, however the hippocampus does receive an indirect connection from the mPFC through a thalamic relay (Vertes, 2006). In the context of emotional memory processing, under baseline conditions the hippocampal, mPFC, and amygdala limbic circuit work in tandem to integrate and process contextual and affective information to orchestrate memory formation and appropriate responses. These reciprocal limbic connections are illustrated in Fig. 1. As discussed earlier, this limbic network is disrupted in PTSD, and is highly sensitive to stress. Repeated or prolonged stress causes plastic and adaptive changes in these structures in preclinical models, which may provide insight into changes and consequences that manifest in the PTSD brain.

Strongly implicated in PTSD, the hippocampus is one of the most widely studied and well-understood limbic structures that has long known to be involved in stress and learning and memory (Kim & Diamond, 2002). As a target and regulator of the stress response, the hippocampus is rich in GC receptors (GRs; Gerlach & McEwen, 1972) that close a negative feedback loop, stopping further GC production. Chronic stress downregulates the levels of GRs, resulting in disrupted negative feedback of the HPA axis, contributing to allostatic load (McEwen, 2007). Chronic stress or chronic GC exposure also causes dendritic retraction within hippocampal subregions, including the CA3 region (Woolley *et al.*, 1990; Watanabe *et al.*, 1992; Conrad *et al.*, 1999; Hoffman *et al.*, 2011), CA1 area, and dentate gyrus principal neurons (Sousa *et al.*, 2000). These

structural changes tend to correspond to behavioral deficits in hippocampal-mediated spatial learning and memory tasks following chronic stress (Conrad *et al.*, 1996; Conrad, 2006; 2010; Hoffman *et al.*, 2011). These effects appear to be transient in nature (Conrad *et al.*, 1999; Sousa *et al.*, 2000; Hoffman *et al.*, 2011), emphasizing the hippocampus as an exquisitely plastic and dynamic structure with potential implications in stress resilience, exhibited and discussed in chapter 2.

Another dynamically plastic structure involved in stress and cognition that is implicated in PTSD is the mPFC. The mPFC is a more recently evolved forebrain structure that is involved in executive function and behavioral control (Miller & Cohen, 2001), and also provides negative feedback to the HPA axis (Diorio *et al.*, 1993). Dendritic retraction has been observed within both the prelimbic (PL) and infralimbic (IL) subregions within the mPFC (Cook & Wellman, 2004; Brown *et al.*, 2005; Izquierdo *et al.*, 2006). Similar to the hippocampus, chronic stress causes reversible dendritic retraction in the mPFC in adulthood (Goldwater *et al.*, 2009; Bloss *et al.*, 2010). These structural alterations also correspond with behavioral deficits mediated by this structure, including impaired working memory (Hoffman *et al.*, 2011; Mika *et al.*, 2012), disrupted behavioral flexibility (Liston *et al.*, 2006; Dias-Ferreira *et al.*, 2009), impaired behavioral inhibition (Mika *et al.*, 2012), and poor fear extinction retention (Miracle *et al.*, 2006; Baran *et al.*, 2009; Hoffman *et al.*, under review). The effects of chronic stress on fear extinction memory, a mPFC mediated behavior, will be demonstrated and discussed in chapter 3.

While the hippocampus and mPFC demonstrate dynamic changes in response to chronic stress, with structural plasticity in dendritic retraction that recovers over time, the

amygdala is another target of stress within the limbic system that shows contrasting patterns of plasticity in response to chronic stress. The amygdala is a phylogenetically old limbic brain structure, that is involved in the encoding and processing of emotionally salient information (LeDoux, 2003), and HPA axis excitation (Herman *et al.*, 2005). In contrast to the hippocampus and mPFC, chronic stress causes dendritic hypertrophy within the amygdala (Vyas *et al.*, 2002; Vyas *et al.*, 2004), with longer durations of stress showing enhanced synaptic connectivity (Vyas *et al.*, 2006). Chronic stress also induces neurophysiological changes in amygdala principal neurons, including hyperexcitability in the lateral amygdala (Rosenkranz *et al.*, 2010). These stress-induced structural and physiological changes correspond to changes in emotionally-laden behavior including increases in anxiety-like behaviors (Vyas *et al.*, 2002), facilitated acquisition of fear learning (Conrad *et al.*, 1999; Hoffman *et al.*, 2010), and resistance to fear extinction (Izquierdo *et al.*, 2006; Hoffman *et al.*, under review). In contrast to the dynamic nature of structural changes observed within hippocampus and mPFC, the dendritic hypertrophy within the amygdala tends to be persistent and does not recover within the same timeframe (21d; Vyas *et al.*, 2004). Furthermore, it has been shown that animals given time to recover (7d) following chronic stress show enhanced fear memories compared to nonstressed controls (McGuire *et al.*, 2010). These consequences could be portrayed as maladaptive plasticity when considering the role of these observed changes within the amygdala in the development of cognitive and emotional psychiatric conditions including PTSD. Chapters 3 and 4 highlight current research that demonstrates a PTSD-like phenotype following chronic stress and fear conditioning, reflecting heightened amygdala processing during fear memory processing.

Dynamic changes in these limbic structures in response to repeated stress in rodent models that overlap with differences observed in the PTSD brain provide a preclinical approach to study the underlying neurobiology of exaggerated fear. Therefore, by manipulating chronic stress in animal models, we can create a brain that has similar characteristics to those that may be predisposed to develop symptomology after a traumatic experience to help us understand mechanisms underlying PTSD.

We summarize a series of experiments aimed to further elucidate chronic stress effects on behavioral, structural, and functional plasticity within these principal limbic structures implicated in PTSD. The overarching hypothesis in this dissertation is that chronic stress induces structural and functional alterations in central limbic structures that create a vulnerability to develop maladaptive fear following a traumatic event. In the absence of the traumatic fear, the individuals can recover and while not completely similar to the non-stressed control condition, there is evidence that these individuals show some benefit as measured by spatial cognition. This is revealed in chapter 2, where we were interested in how a post stress recovery period would impact hippocampal-dependent spatial reference and working memory, and its relation to hippocampal structural changes. In contrast to the study in described in chapter 2, individuals exposed to a traumatic event, as modeled in a fear conditioning paradigm with tone-footshock presentations, the outcome might be maladaptive. Chapter 3 describes a study where we were interested in how chronic stress affects nonassociative contextual fear and generalization during auditory fear extinction. We also asked how chronic stress impacts fear extinction memory-induced functional activation within the hippocampus, mPFC, and amygdala. Finally in chapter 4, we conducted a series of studies to determine chronic

stress and fear conditioning effects on functional activation within amygdala and hippocampal subregions during memory reactivation, and whether chronic stress enhanced fear memories are vulnerable to disruption by manipulating memory reconsolidation in a novel attempt to weaken stress induced strong fear memories. Utilizing a variety of methods to investigate behavioral, morphological, and functional activation alterations in stress- and PTSD-associated neural substrates following chronic stress, we aim to have a better understanding of the neurobiological consequences of chronic stress and maladaptive fear.

CHAPTER 2

RECOVERY AFTER CHRONIC STRESS WITHIN SPATIAL REFERENCE AND

WORKING MEMORY DOMAINS: CORRESPONDENCE TO HIPPOCAMPAL

MORPHOLOGY

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Chronic stress causes behavioral impairments in hippocampal-dependent spatial

learning and memory (Luine *et al.*, 1994; Wright & Conrad, 2005; Conrad, 2010), which

often correspond with hippocampal CA3 dendritic retraction (Watanabe *et al.*, 1992;

Magarinos & McEwen, 1995; Vyas *et al.*, 2002; Conrad, 2006; McLaughlin *et al.*, 2007).

Moreover, these alterations within hippocampus-dependent behavior and morphology can

recover. For example, chronic stress-induced spatial learning deficits on a radial arm

maze task recovered 18 days after the end of restraint (Luine *et al.*, 1994). Similarly, CA3

dendritic retraction following chronic restraint reversed within 10 days after stress ended

(Conrad *et al.*, 1999). These data suggest strong ties between hippocampal morphology

and function; however, the work was carried out in separate cohorts of animals.

One study investigated whether chronic stress caused spatial learning deficits and

hippocampal dendritic retraction within the same animals using a Morris water maze task

(Sousa *et al.*, 2000). Chronic stress for four weeks impaired spatial learning and produced

hippocampal dendritic retraction, and both recovered within a month following the end of

stress. While important, the study by Sousa and colleagues (2000) raises additional

questions. For instance, the behavioral deficit effect was relatively mild, occurring briefly

at the midpoint of testing and without an effect on the spatial memory retention probe

trial. The lack of an effect on the probe trial may be attributed to the rats performing well

by the last acquisition trial and having retained the information, or, perhaps the opportunity was missed to assess strategy when groups differed. Differences may also reflect a stress-induced switch to a non-spatial strategy from prolonged training (Packard & McGaugh, 1996; Sandi & Pinelo-Nava, 2007), and/or from chronic stress causing a shift to habit-based strategies (Dias-Ferreira *et al.*, 2009). Additionally, the relatively moderate deficit occurred despite extensive dendritic retraction throughout several hippocampal subregions. Consequently, it remains to be determined whether chronic stress that has been established to produce hippocampal CA3 dendritic retraction (Conrad, 2006), would be effective at impairing spatial search strategies during periods of training when deficits exist and whether these effects recover.

The current study implemented a modified version of the two-day radial arm water maze (RAWM, Alamed *et al.*, 2006; Diamond *et al.*, 2006), an aversive spatial task that combines aspects of the Morris water maze and a radial arm maze. The advantage of the RAWM is that each trial is essentially a probe trial, allowing one to assess two types of spatial function: reference and working memory. Therefore, the current study investigated whether chronic stress impaired spatial ability during acquisition and/or retention, whether these effects were carried by the domains of spatial reference or working memory, whether these behavioral metrics corresponded with CA3 dendritic complexity immediately after chronic stress ended, and finally, whether the behavioral and morphological outcomes recovered after a post-stress delay.

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Method

Subjects

Twenty-seven five-month-old male Sprague-Dawley rats from the colony of the National Institute on Aging at Harlan Laboratories (Indianapolis, IN) were pair housed on a 12:12 light cycle. Food and water were available *ad libitum* except during restraint procedure. Rats were given one week to acclimate before the start of any procedures. Rats were weighed weekly throughout all experimental manipulations. All testing was conducted during the light phase of the light cycle. The procedures followed the Guide for Care and Use of Laboratory Rats (Institute of Laboratory Animal Resources on Life Science, National Research Council, 1996) and were approved by the Institutional Animal Care and Use Committee at Arizona State University.

Group assignments

Rats arrived at 5 months of age and were randomly divided into one of three groups (n=9/group): non-stressed control (CON), chronically stressed and tested immediately (STR-IMM), and chronically stressed then given a 21d post stress delay then tested (STR-DEL). Groups and procedures are outlined in Fig. 2.

Chronic restraint procedure

We were interested in testing spatial abilities at the earliest time point that CA3 dendritic morphological changes occur. Thus, we implemented a chronic restraint procedure that involves 6h restraint/day for 21d because it produces hippocampal CA3 dendritic retraction that persists for four days after chronic restraint ends (Conrad *et al.*, 1999) and recovers by 10 days from the end of restraint (Conrad, 2006; McLaughlin *et al.*, 2007). During designated restraint periods for the STR-IMM and STR-DEL groups,

rats were transported in their home cages to a different room and restrained in wire mesh restrainers for 6h/d/21d. Wire mesh restrainers were 18cm circumference x 24cm long (wire mesh from Flynn and Enslow Inc, San Francisco, CA) with wire ends sealed with grip guard sealer (ACE Hardware); larger restrainers (23cm circumference x 28cm long) were used for rats that outgrew the smaller restrainers. After the acclimation period, STR-DEL rats were restrained for 21d and then given a 21d delay after the restraint procedure before testing, whereas the STR-IMM rats were undisturbed during the first 21d and restrained during the second 21d, but tested the day after restraint ended. Therefore, all animals were age-matched during behavioral testing and sacrificed (at 7 months of age). Nonstressed controls and rats not undergoing restraint had free access to food and water during periods that other rats were restrained because we have shown that food restriction for stressed and control groups does not affect cognitive or morphological outcomes (Kleen *et al.*, 2006). Fig. 1 illustrates the timeline of the study.

Radial arm water maze (RAWM)

Apparatus

The RAWM was constructed of black polypropylene and consisted of eight symmetrical arms (27.9 cm long x 12.7 cm wide), radiating outward from a center annulus (diameter, 48 cm), and filled with water (19° C) rendered opaque from powder black tempera paint. Within one of the arms, a platform was located at the end and submerged 2.5 cm below the surface of the water. The platform was in a constant location for all trials for a given rat, and the location was counterbalanced across rats to control for any potential location effects. Testing was conducted in a room containing numerous distal visual cues outside the maze that remained constant throughout testing.

RAWM Procedure

Testing in the RAWM took place over two consecutive days, between 0800 and 1600 h and was based upon the parameters described by (Alamed *et al.*, 2006). On the first day of testing, each rat received 12 massed trials, followed by a one-hour delay spent in testing cages, and concluded with 6 additional training trials. The next day, all rats were given a single retention trial. A trial began when a rat was released into an arm that did not contain the platform (the start arm) and was given 3min to locate the hidden platform. The start arm varied across trials within a day, but was never located directly across from the platformed arm. If the rat failed to locate the platform within the allotted time, the rat was manually guided to the platform, allowed to remain on the platform for 15s, and then returned to the home cage for 15s. A net was used to stir the water between trials in the maze and sweep it clean of bedding and feces to prevent rats from using potential nonspatial search strategies. An arm entry was recorded when the tip of the rat's nose reached 11cm into an arm. First time entries into a non-platformed arm were recorded within the domain of reference memory errors, and repeat entries into non-platformed arms within a trial were recorded within the domain of working memory errors. Errors were scored for each trial throughout acquisition and retention phases.

Staining Procedures

Within one hour of completing the RAWM, rats were deeply anesthetized with isoflurane, decapitated, and then unperfused brains were rapidly removed. FDRapid Golgistain™ kits (FD NeuroTechnologies, Baltimore, MD, USA) were used for Golgi staining. Brains were immersed in solution AB (made 24h prior to brain immersion) as per instructions of the manufacturer. The AB solution was replaced after the first 12h.

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Brains were kept in AB solution for 3wk in a dark location, and then were transferred to solution C for 1wk. For sectioning, brains were blocked to target the hippocampus, frozen in 2-methylbutane and cut (100μm thickness, coronal sections, Microtome HM 500 OM Cryostat, 25/30 °C). Once placed on a subbed slide, brains were firmly secured in place by hand pressure against Bibulous blotting paper (Fisher Scientific International Inc., USA). Slides were left to dry for 1d in a dark location before staining. Slides were rinsed in distilled water and placed in the developing solution for 10min. After developing, slides were rinsed in distilled water, dehydrated in ascending series of ethanol, cleared in neoclear (Harleco®, Gibbstown, NJ) and coverslipped with Permount Mounting Media (Fisher Scientific International Inc.). Slides were left in the dark to dry for 1–2wk.

Neurons were chosen based on the following criteria: 1) the cell body and dendrites were fully impregnated and untruncated, 2) the cell was relatively isolated from surrounding neurons, and 3) the cell was located in the CA3 region of the hippocampus. A camera lucida drawing tube attached to an Olympus BX51 microscope was used to trace all neurons (320x). Dendritic length was quantified using a Scale Master II digital plan measuring system (Calculated Industries, Inc., Carson City, NV) linked with a PC interface to a Dell PC. CA3 neurons were further labeled as short-shaft (SS) or long-shaft (LS) depending on their relative location in the stratum pyramidal and proximal apical shaft length. The apical dendrites of the SS neurons in the CA3 region are intrinsically more complex than the apical dendrites of the LS neurons, so the two values for the SS and LS neurons were averaged to obtain one value for each rat. For a rat to be included in the analysis, the brains must have contained at least three successfully stained neurons of each SS and LS categories (6 neurons/animal). Dendritic length and branch points

(number of dendritic bifurcations) were quantified for apical and basal sections of the SS and LS neurons.

Statistical Analysis

Data for RAWM on day 1 were analyzed by blocks of two trials. For instance, trials 1 and 2 of acquisition were averaged and represented as block 1. Data were analyzed by analysis of variance (ANOVA) and when significant effects were detected at a *p*-value of .05 or less, Newman-Keuls post hoc tests were performed. Data are represented by means ± S.E.M. with 5-6 subjects per group for RAWM analyses, 6-7 subjects per group for hippocampal dendritic morphology analyses, and 9 per group for body weight gain analyses. In addition to calculating omnibus *F*-values, we also conducted two planned comparisons for repeat entry errors during the retention trial in the RAWM: between CON and STR-IMM; and between STR-IMM and STR-DEL. For the 24h retention trial, comparisons between CON and STR-DEL were made using analysis of covariance (ANCOVA), with covariance for the mean of the first 12 trials of acquisition for both first time and repeat entry errors. These analyses were performed in order to reduce error variance during training on day 1 in the RAWM. RAWM testing was performed on all animals (N=27, n=9/group), however given the high physical demand of the task, some of the animals did not complete the task and therefore were not included in final analyses. Criteria to exclude an animal in RAWM analyses was 2 or more trials (out of 19) in which the animal stopped exploring the maze. Animals per group included in behavioral analyses are as follows: CON (n=5), STR-IMM (n=5), STR-DEL (n=6).

Results

Radial arm water maze (RAWM)

Acquisition

All groups decreased first time entry errors across the first six blocks (12 trials) of training in the RAWM without any treatment effects (Fig. 3A). Indeed, a mixed factors ANOVA for stress history across blocks 1-6 revealed a main effect of block, $F(5,65)=5.519$, $p<.001$. There were no other main effects or interactions with stress history, demonstrating that control and both stressed groups showed similar learning rates for the reference memory domain.

All groups decreased repeat entry errors across the first six blocks (12 trials) of training in the RAWM (Fig. 3B). A mixed factors ANOVA for stress history across blocks 1-6 revealed a significant main effect of block, $F(5,65)=3.401$, $p<.01$. There were no other significant effects, which demonstrates that all groups showed comparable spatial working memory performance during acquisition of the task.

Short term memory retention: 1h delay and relearning

Following the first twelve trials (six blocks) of RAWM acquisition, all groups were given a 1h delay in the same testing room, followed by an additional six trials (three blocks) of training. All groups exhibited significantly more first time entry errors following the 1h delay compared to the last block of acquisition (Fig. 3A). A mixed factors ANOVA for stress history across blocks 6 and 7 revealed a main effect of delay, $F(1, 13)=16.06$, $p=.001$, with errors increasing after the delay. There were no other significant main effects or interactions with stress history, which again demonstrate comparable performance among groups for the reference memory domain.

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A mixed factors ANOVA (stress history x block) across blocks 7-9 revealed a main effect of block, $F(2, 26)=3.873$, $p<.05$, representing that all groups decreased first time entry errors across the relearning period (Fig. 3A). There were no other significant effects. Therefore, the controls and both stress groups showed similar learning rates for the reference memory domain after a 1h delay.

All groups exhibited similar levels of repeat entry errors following the 1h delay compared to the last block of acquisition (Fig. 3B). Moreover, all groups showed similar repeat entry errors across the last three training blocks (Fig. 3B).

Long term memory retention: 24h

After the completion of RAWM training, all groups were given a 24h delay followed by a single retention trial to investigate long term memory, which revealed significant treatment effects. A mixed factor ANOVA for stress history with time as a repeated measure (block 9 and retention trial) revealed a significant effect of time, $F(1, 13)=5.434$, $p<.05$. Rats made more errors on the retention trial than they did in block 9, the last training block 24 hours earlier. However, when inspecting long term memory retention for each group, chronically stressed rats given a delay (STR-DEL) made fewer errors, while the other two groups (STR-IMM, CON) exhibited increased errors during the retention trial than the last acquisition block (block 9). Interestingly, the chronically stressed rats that were tested immediately (STR-IMM) showed similar first time arm entries during retention (3.4±1.0) as they did on the first training block (2.7±0.4), as if the prior acquisition trials had not taken place (comparing Block 1 on Fig. 3A to the retention trial). Analysis of the retention trial alone using a one-way ANOVA for stress history and first time entry errors showed a significant main effect of stress history, $F(2, 15)=3.584$,

p=.05. Post hoc analyses revealed that when chronically stressed animals were tested immediately after the stress period (STR-IMM), they exhibited significantly more errors of the reference memory domain during the retention trial compared to the stressed rats that were given a 21d delay after chronic stress (STR-DEL). To probe the 24h retention trial further, additional analyses were performed using ANCOVA with the mean of the first 12 trials (six blocks) of testing as a covariate in order to reduce error variance from training to compare CON and STR-DEL. Since the covariate (the first 12 trials of acquisition) occurred after the stress manipulations had taken place, we tested the homogeneity of regression, and found the interaction between the covariate and stress history to be non-significant, $F(2, 16)=2.582$, $p>.05$, with a medium effect size ($Eta^2=.089$), thereby justifying the use of ANCOVA. The ANCOVA revealed a significant effect of stress history, $F(1, 11)=26.417$, $p<.001$, illustrating that STR-DEL had fewer first time entry errors than CON during retention 24h after RAWM training (Fig. 3C).

For repeat entry errors, the omnibus ANOVA comparison of the 24h retention trial to the last block of training revealed no statistical differences in that all groups showed similar levels of repeat entry errors for both test periods. However, this effect was likely carried by the STR-DEL group and the CON group, which both had almost no errors during the retention trial. A repeated measures ANOVA for repeat entry errors across block 9 and the retention trial showed a significant main effect of stress history, $F(2,15)=3.98$, $p<.05$, with a post hoc analyses revealing that chronically stressed rats that were tested immediately (STR-IMM) exhibited significantly more repeat entry errors compared to the other two groups (Fig. 3B). Additionally, planned comparisons for

repeat entry errors during the retention trial revealed that when animals were chronically stressed and tested immediately after, they made significantly more errors compared to nonstressed controls and chronically stressed rats with a 21d delay, (CON vs. STR-IMM, t=1.897, p<.01; STR-IMM vs. STR-DEL, t=21.976, p=.001). Notably, repeat entry errors for the STR-IMM group were the highest after a 24h delay during the retention trial (2.6 ± 1.2) when compared to any of the training blocks (≥1.2 ± 0.62). An ANCOVA was also performed comparing CON and STR-DEL for repeat entry errors during the 24h retention trial with the mean of the first 12 trials (6 blocks) of testing as a covariate; this analysis yielded no statistical differences (Fig. 3D). No other effects were significant.

Hippocampal CA3 dendritic retraction

Brains were processed approximately 1 hour after the retention trial in the RAWM. Thus, this was 2d after chronic stress ended for the STR-IMM group, and 23d after chronic stress ended for the STR-DEL group. For rats that were tested and sacrificed soon after the duration of restraint, chronic stress (STR-IMM) caused CA3 apical dendritic retraction compared to nonstressed controls, and CA3 dendritic retraction recovered several weeks after chronic stress had ended (STR-DEL). Two separate one-way ANOVAs (one for branch points and one for branch length) revealed a significant main effect of stress history on apical branch points, $F(2, 19)=5.295$, $p<.05$, and apical branch length, $F(2, 19)=4.398$, $p<.05$. Post hoc analyses revealed that STR-IMM had reduced apical branch points (Fig. 4A) and apical branch length (Fig. 4B) compared to CON ($p<.05$ for both) and STR-DEL ($p<.05$ for both). The CON and STR-DEL groups were statistically similar to each other. For the CA3 basal region, the three treatment groups were statistically similar (Fig. 4C and 3D, respectively). Representative

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photomicrographs of hippocampal CA3 neurons from CON, STR-IMM, and STR-DEL groups in Fig. 4E-G (top panel), respectively, (and Camera Lucida drawings in respective Fig. 4E-G, bottom panel) illustrate these effects of stress-induced apical dendritic retraction. These images (Fig. 4E-G) represent dendritic properties of each experimental group. Note the decrease in apical dendritic arbors in the STR-IMM group compared to both CON and STR-DEL groups. Also note the restoration of apical dendritic complexity in the STR-DEL group to levels of CON.

Body weight

Analyses of body weight gain throughout the study confirmed chronic restraint as an effective stressor and that partial recovery occurred following a 21-d delay (Fig. 5). A one-way ANOVA for stress history on body weight on day 1 showed no differences among the groups. A mixed factors ANOVA for stress history across weeks revealed a significant effect of week, $F(5, 120)=15.754$, $p<.001$, a significant stress history by week interaction, $F(10, 120)=129.651$, $p<.001$, and a significant main effect of stress history, $F(2, 24)=14.217$, $p<.001$. Post hoc analyses revealed significant differences between CON and STR-IMM as well as a significant difference between CON and STR-DEL. CON rats gained weight steadily throughout the experiment. As expected, STR-IMM rats gained weight for the first three weeks, when they were not restrained, and then showed weight loss during restraint from weeks three to six. In contrast, STR-DEL rats lost weight in the first three weeks and then gained weight during the last three weeks.

Discussion

The present study is the first to evaluate the reversibility of chronic stress-induced deficits on hippocampal-dependent spatial reference and working memory domains "on-

line" as the rats navigated, and whether these changes corresponded to hippocampal CA3 dendritic retraction within the same animals. While chronic stress increased first time and repeat entries during retention testing on the RAWM, and resulted in hippocampal CA3 dendritic retraction, rats that had the opportunity to recover from the chronic stress experience showed no evidence of a detriment on maze performance, nor perturbation of CA3 dendritic complexity. Indeed, chronically stressed rats given a 21d recovery period were better than non-stressed controls in the domain of reference memory. Therefore, these findings show that chronic stress produces deficits on two domains of memory function: spatial reference memory (first time entry errors) and spatial working memory (repeat entry errors), and that these functional deficits correspond to the presence of CA3 dendritic retraction, with both function and morphology recovering after chronic stress ended. However, the lasting beneficial effects following chronic stress on spatial reference memory were independent of CA3 dendritic complexity.

Changes in hippocampal CA3 neuronal dendritic complexity have been proposed to underlie hippocampal spatial ability, but with some caveats. Our model of chronic stress (wire mesh restraint 6h/d/21d) has produced reliable hippocampal CA3 apical dendritic retraction (Watanabe *et al.*, 1992; Conrad *et al.*, 1999; McLaughlin *et al.*, 2007), which also results in impairments in spatial learning and memory (Luine *et al.*, 1994; Conrad *et al.*, 1996). We demonstrate here that chronic stress produces hippocampal-dependent memory deficits in two domains of memory, and that these deficits correspond with hippocampal CA3 dendritic retraction within the same animals. Interestingly, the parallels between CA3 dendritic morphology and function were based upon rats showing CA3 dendritic retraction and spatial memory deficits, but did not correspond to rats that

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had recovered from chronic stress and showed better spatial ability compared to controls. Indeed, there have been some anomalous findings where chronic stress produced CA3 dendritic retraction with uncompromised spatial ability. These findings were observed with chronic corticosterone treatment (Luine *et al.*, 1993; Bardgett *et al.*, 1996; Sousa *et al.*, 2000), in females (Bowman *et al.*, 2001; Conrad *et al.*, 2003; McLaughlin *et al.*, 2005), and in a contextual fear conditioning task (Conrad *et al.*, 1999; Sandi *et al.*, 2001). Collectively, these studies illustrate that chronic stress-induced CA3 dendritic retraction contributes, but is not the exclusive factor in hippocampal learning and memory ability and that an increase in dendritic complexity beyond baseline levels is not necessary for improved performance beyond control levels.

Our findings that chronically stressed rats recovered from the deficits in spatial ability and hippocampal dendritic retraction corroborate other work, but with important differences among the findings. Similar to previously published data (Luine *et al.*, 1994; Sousa *et al.*, 2000), when animals were given a delay period following chronic stress prior to behavioral testing, stress-induced hippocampal-mediated behavioral deficits recovered. However, our results differ from Sousa et al. (2000), in that Sousa and colleagues showed stress-induced deficits midway through acquisition without retention deficits in the Morris water maze, whereas we found deficits during 24h retention in the RAWM without acquisition impairments. The disparity between the two effects could be attributed to several differences. First, Sousa and colleagues (2000) showed a modest learning impairment on just two trial blocks out of a 12d paradigm, which may relate to arousal and amygdala activation. The amygdala is engaged when rats learn a stressful water maze task and perform well (Akirav & Richter-Levin, 1999; Kim *et al.*, 2001; Kim

et al., 2005), and failure to activate the amygdala results in poor water maze performance (Akirav *et al.*, 2001). Since water mazes are inherently stressful and engage the amygdala, then deficits may be hard to detect in the first days of learning, which is corroborated by a review of chronic stress effects on various types of spatial tasks (Conrad, 2010). The work from Sousa and colleagues (2000), our own study and another study using a different version of the RAWM (Park *et al.*, 2001) did not find differences in the first day or so of testing. But as the rats were re-exposed to the water maze, then Sousa and colleagues (2000) and Park et al., (2001) detected acquisition deficits, whereas rats in our study were not given multiple acquisition days. A second difference is that Sousa and colleagues tested rats over twelve days, which could have overlapped with the time frame that the hippocampus recovered, such as the changes in CA3 dendritic retraction (Conrad *et al.*, 1999). Or, the multiple training days could have produced over-training and allowed the rats to move to habit-based strategies rather than using spatial strategies (Packard & McGaugh, 1996; Dias-Ferreira *et al.*, 2009).

Also notably, the model of chronic stress differed across these studies. Unpredictable components were incorporated into the models used by Sousa et al. (2000), who used a heterotypic model with different daily stressors, and Park et al. (2001) who combined daily predator exposure with rotating liter mates. In contrast, our chronic restraint regimen involved the same daily stressor. One interpretation is that the homotypic nature of repeated restraint may have recruited memory-related brain structures that process predictability, such as the prefrontal cortex (Amat *et al.*, 2005; Maier *et al.*, 2006). Regardless of the interpretation, the outcome is consistent with the timeline for recovery following chronic stress and supports the understanding that

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chronic stress does not impair acquisition in early training trials in spatial water mazes, but that deficits can be detected after an early adaptation period. However, it is important to consider whether the observed effects are specific to chronic restraint, or generalizable to other chronic stress paradigms.

Unexpectedly, the chronically stressed rats given the opportunity to recover showed spatial memory retention that was even better than rats left undisturbed, suggesting that chronic stress had lasting effects. During the retention trial 24h following training in the RAWM, rats that were given a 21d delay from the end of chronic stress showed a significant improvement in first time entry errors compared to chronically stressed rats without this post-stress delay (Fig. 3A), and even a reduction in first time entry errors compared to nonstressed controls (Fig. 3C). Indeed, chronically stressed rats with a 21d delay showed fewer errors in the reference memory domain during their 24h retention trial compared to the end of RAWM training. To our knowledge, this study is the first to report that recovery from chronic stress in adult male rats can lead to improved spatial ability. Our findings correspond with previous studies in humans, non-human primates, and rodents, as they have reported that early stressful experiences impact the brain and behavior when evaluated later in life (Eiland & McEwen, 2012) As examples, early life stress alters the development of neural networks (Ito *et al.*, 1998) and promotes psychological and physiological adaptation and/or resilience to stressors in adulthood (Levine, 1957; Levine *et al.*, 1957; Meaney *et al.*, 1988; Charney, 2004; Lyons *et al.*, 2010). Underscoring the potentially lasting effects of stress, a stressful procedure in pups involving briefly separating them from their dam prevented hippocampal CA3 dendritic retraction when chronic restraint was administered in adulthood (Eiland & McEwen,

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2012). A recent review suggests that some forms of stressors during early development and childhood may influence the hippocampus favorably, allowing an organism to respond well to aversive tasks later in life (Champagne *et al.*, 2008) and our current findings reveal that chronic stressors in adulthood may have some potentially beneficial effects for future stressful experiences.

A potential mechanism mediating the facilitation of spatial memory following a 21d recovery period after chronic stress involves the amygdala-hippocampal relationship. Hippocampal-dependent spatial memory is modulated by amygdala activation (Akirav & Richter-Levin, 1999; Akirav *et al.*, 2001; Kim *et al.*, 2001; Kim *et al.*, 2005), and the amygdala can become engaged with tasks that are highly arousing. The aversive component of water mazes is thought to engage the amygdala and increase levels of endogenous glucocorticoids (Sandi *et al.*, 1997; Conrad, 2010). Furthermore, changes in dendritic structure may contribute to functional outcomes (Conrad, 2006). Specifically, the current and other studies document that the CA3 region of the hippocampus recovers from dendritic retraction following chronic stress using restraint or immobilization (Vyas *et al.*, 2004). Unlike the hippocampus, the basolateral region of the amygdala expresses dendritic hypertrophy that persists when the CA3 region of the hippocampus recovers (Vyas *et al.*, 2004). Consequently, the still hypertrophied amygdala may express strengthened connections to the restored hippocampus to facilitate spatial memory. Whether the amygdala restores to pre-stress dendritic complexity beyond the 21d post-stress period is unknown (Vyas *et al.*, 2004), but this potential mechanism underlying enhanced hippocampal-dependent spatial memory warrants future investigation.

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In conclusion, the current report is the first study to show that chronic stress in adult rats can have immediate impairing effects on the domains of spatial reference and working memory, and delayed beneficial effects on spatial reference memory. When chronic stress produced CA3 dendritic retraction, deficits were detected in the domains of spatial reference and working memory. However, when stress-induced CA3 dendritic retraction recovered, spatial performance not only improved, but spatial reference memory was better than the non-stressed controls. This ability for chronically stressed rats to recover and even show resilience when confronted with a challenging cognitive task has also been described in chronically stressed rat pups when tested on cognitive ability later as an adult (Meaney *et al.*, 1988). Clearly, chronic stress can have detrimental effects on hippocampal morphology and function (Conrad, 2006; 2010), but the current findings reveal that chronic stress initiated in the adult can also have potentially beneficial outcomes.

CHAPTER 3

CHRONIC STRESS DISRUPTS FEAR EXTINCTION AND ENHANCES

AMYGDALA AND HIPPOCAMPAL FOS EXPRESSION IN AN ANIMAL MODEL

OF POST TRAUMATIC STRESS DISORDER

Currently under review

Post traumatic stress disorder (PTSD) is a debilitating and increasing public

health problem, especially in combat-exposed populations. The lifetime prevalence of

PTSD in the United States has been reported to be ~6% (Kessler *et al.*, 2012). PTSD

develops in a subset of those experiencing a traumatic event (Breslau *et al.*, 1991), which

suggests individual differences in the susceptibility and resilience to the development of

the disorder after trauma exposure. One biological risk factor that has been identified for

PTSD is reduced hippocampal volume (Gilbertson *et al.*, 2002). Furthermore, functional

imaging studies in PTSD patients reveal heightened amygdala responsivity, as well as

reduced volume and responsivity within the medial prefrontal cortex (mPFC), and

reduced volume and neural integrity within the hippocampus (Shin *et al.*, 2004; Shin *et

al.*, 2006). Although these observed regional changes provide putative neural substrates

for PTSD research, whether these alterations are contributing factors to, or outcomes

from the disorder is unknown.

Animal models can help approach questions raised in clinical research in

prospective designs under controllable conditions. Chronic stress leads to structural and

behavioral alterations in rodents that parallel the changes observed in humans with

PTSD. Within the amygdala, chronic stress causes dendritic hypertrophy (Vyas *et al.*,

2002; Vyas *et al.*, 2004; Padival *et al.*, 2013) and hyperexcitability (Rosenkranz *et al.*,

2010). These stress-induced structural and physiological changes correspond to changes in emotionally-laden behavior including increases in anxiety-like behaviors (Vyas *et al.*, 2002), facilitated acquisition of Pavlovian fear learning (Conrad *et al.*, 1999; Sandi *et al.*, 2001; Hoffman *et al.*, 2010), and resistance to fear extinction (Izquierdo *et al.*, 2006). In contrast to the amygdala, chronic stress causes dendritic retraction within the hippocampus (McLaughlin *et al.*, 2007) and medial prefrontal cortex (mPFC; Cook & Wellman, 2004; Brown *et al.*, 2005) that correspond to impaired spatial learning and memory (Conrad, 2010; Hoffman *et al.*, 2011) and impaired fear extinction retention (Miracle *et al.*, 2006; Baran *et al.*, 2009). Therefore, manipulating chronic stress in animal models allows for the induction of neural and behavioral changes that are similar to those that may predispose individuals to develop symptomatology.

Pavlovian fear conditioning is a widely used model to study the neurobiology of fear and PTSD. In these paradigms, a neutral stimulus (such as a tone) serves as the conditioned stimulus (CS) and is paired with an aversive stimulus (such as a footshock)—the unconditioned stimulus (US). The animal learns the association between CS and US, and exhibits a conditioned response (CR, such as freezing) in the presence of the CS. Analogous to exposure therapy in humans, a common PTSD treatment approach, fear extinction occurs with repeated unreinforced CS presentations that result in a new, inhibitory memory trace, or a CS-no US association. One challenge with PTSD populations is the relapse of symptoms between extinction sessions, i.e., fear responding recovers between exposure therapy sessions and outside the therapy context (discussed in Hamner *et al.*, 2004). Whereas previous work has shown that chronic stress impairs the recall of extinguished cued fear (Miracle *et al.*, 2006; Baran *et al.*, 2009), it is unknown

how a history of chronic stress impacts nonassociative fear, such as in the absence of the CS or in a novel context (Kamprath & Wotjak, 2004), which is pertinent to the hyperarousal symptom cluster in PTSD patients (Yehuda & LeDoux, 2007). Furthermore, how the chronically stressed brain becomes engaged during the retrieval of a fear memory has been virtually unexplored. The current study aimed to investigate (1) how a history of chronic stress impacts both cued and context extinction following cued fear conditioning, (2) how chronic stress affects fear responding in a novel context following extinction, and (3) how chronic stress impacts functional activation of limbic structures involved in fear learning and extinction during retrieval of a cued fear memory.

Method

Subjects

Twenty male Sprague-Dawley rats weighing approximately 250-275g upon arrival (approx. 2 months old; Charles River Laboratories) were pair-housed in light and sound attenuating chambers (21-22°C) on a 12:12 reverse light cycle (lights off at 6am) according to conditions specified by the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources on Life Science, National Research Council, 1996). Food and water were available *ad libitum* except during restraint procedures (described below). All procedures occurred during the dark phase of the light cycle.

Prior to group assignments, all animals were tested in a single open field (OF) for anxiety-like behavior and locomotion profiles. OF testing was consistent with our previously published procedures (Huynh *et al.*, 2011) and helped to distribute similar profiles across groups (Bellani, Luecken, & Conrad, 2006). Briefly, animals were placed

at pseudorandom locations in an open square arena (110cm x 110cm, 30cm height) under low light intensity (200lx) and given 10min to explore then returned to their home cage. The OF arena was cleaned after each trial with pet deodorizer. OF behavior was recorded using an overhead video camera for offline scoring. Behavior was scored using 1) grid crossings, defined as the front two paws traversing a center or peripheral grid line, and 2) center grid time, recorded from the time the front two paws crossed the center grid until the front two paws exited the center grid.

Following OF testing, animals were divided into non-stressed control (CON) or chronically stressed groups (STR), n=10/group, and further subdivided into subgroups for the same and novel context testing condition (described below). All groups had similar locomotor and anxiety-like behavior profiles in OF (data not shown).

Stress Manipulation

Rats were chronically stressed via repeated wire mesh restraint (STR) or not (CON), and were weighed weekly. During the designated restraint period, STR rats were restrained in their home cages in wire mesh restrainers for 6h/d/21d. Wire mesh restrainers were 18cm circumference x 24cm long (wire mesh from Flynn and Enslow Inc, San Francisco, CA) with wire ends sealed with grip guard sealer (ACE Hardware). CON rats were handled briefly each day, with their food and water restricted while the STR rats were restrained to keep food and water access similar across treatment conditions.

Fear Conditioning: Apparatus

Rodent fear conditioning chambers (25 cm depth x 29 cm height x 26 cm width: Coulbourn Instruments, E10-18TC) were contained in sound-attenuating cubicles

(Coulbourn, E10-23, white). A PC interface card (Coulbourn, PCI-3-KIT) adapted to a PC, a universal link (Coulbourn, L91-04S), and Graphic State software (v 3.03 GS3.03) controlled the stimulus presentation. A frequency generator (Coulbourn, E12-01) produced a tone (75 dB, ~3.0 kHz) through a speaker located in the side panel of the conditioning chamber. The shock (500 ms, 0.35mA, Coulbourn Animal Shock Generator, H13-15) was administered as a current, equally distributed through a metal grid floor (Coulbourn, E10-18RF). Behavior was videotaped for off-line analysis using a camera (Coulbourn, E27-91) mounted on the ceiling and a videocassette recorder. Infrared lights were located on the side panels of the chamber to denote the onset and offset of the tone, because no audio was recorded. A house light (Coulbourn, E11-01) was mounted in the side panel to illuminate the chamber at all times.

Two distinct chamber contexts (contexts A and B) were utilized for different fear conditioning testing phases. Context A consisted of white and silver paneled walls, a wire bar shock floor with a white catch pan, and was cleaned with 70% ethanol. Context B consisted of striped paneled walls, a smooth Plexiglas® floor insert and a dark catch pan, and was cleaned with an orange scented cleaner (method® clementine all purpose natural surface cleaner, methodhome.com).

Fear Conditioning: Procedure

During the last two days of restraint stress, all testing groups were transported by cart in their home cage into the fear conditioning testing room and left on the cart for 30min to acclimate to the transport process and room. One day following the end of the stress procedure, rats were acclimated to testing chambers (context A) for 10min with the house light on. The following day they were placed into the conditioning chamber

(context A) and given three-30s tones that co-terminated with a 0.35mA, 500ms shock,

(ITI 120-360s) and were then transported back to their home colony. Over the following

two days (Extinction 1 and 2, respectively), rats were subjected to extinction testing in

context A that consisted of 15 tone-alone trials (ITI 120-360s), which were averaged into

blocks of 3 trials (5 blocks/extinction session). The next day, STR and CON groups were

subdivided and tested (6 tone-alone trials) in either the same context (STR-SAME, CON-

SAME) or a novel chamber context (context B; STR-NOVEL, CON-NOVEL),

n=5/group. An experimental timeline is illustrated in Fig. 6A.

Fear Conditioning: Dependent Variable

Behavior was videotaped for scoring later by observers blind to experimental

conditions. For cued fear conditioning, the dependent variable measured was the number

of seconds freezing during each 30s tone presentation, whereas contextual fear was

defined as the number of seconds freezing during the 30s prior to tone onset. Freezing

was defined as the absence of all movements except those associated with respiration

(Blanchard & Blanchard, 1969; Conrad *et al.*, 1999; Quirk *et al.*, 2000; Baran *et al.*,

2009; Baran *et al.*, 2010; Hoffman *et al.*, 2010). Data analyses were performed on raw

data (number of seconds spent freezing during the 30s tone). However, for clarity of

presentation, these same data will be represented as a percentage of freezing during the

30s tone, or respective context interval. One rat from the control group was eliminated

from behavioral and Fos analyses due to almost no movement during behavioral testing,

leaving n=9 for CON during extinction analyses. Another rat was excluded from the

behavioral results because it was a statistical outlier, as calculated by a Grubb's test, n=4

for CON-SAME data for freezing to CS and context.

Tissue Preparation and Fos immunohistochemistry

To capture peak Fos protein expression (Sonnenberg *et al.*, 1989; Nestler *et al.*, 2001), all rats were overdosed with sodium pentobarbital (100mg/kg, i.p.) 90 minutes following placement in the testing chambers for SAME or NOVEL conditions (described above). Adrenal glands were collected and rats were then transcardially perfused with phosphate buffered saline (pH 7.4) and 4% paraformaldehyde (pH 7.4), and brains were removed and post-fixed in 4% paraformaldehyde and stored at 4°C overnight. Brains were then cryoprotected in 15% and 30% sucrose over 2d, and stored at 4°C until sectioning.

Brains were sectioned on a cryostat at 40μm within two weeks of perfusion. Multiple series of slides were taken at each level of section for separate cresyl violet and immunohistochemistry staining procedures. Sections mounted on slides were then stored at -80°C until tissue processing. One series of slides was stained with cresyl violet to identify and confirm subregions of interest for Fos analysis.

Another series of slides containing subregions of interest were processed for immunohistochemistry against Fos protein, which will be termed Fos-like immunoreactive (Fos-IR) labeling. Target sections were washed three times in 1x phosphate buffered saline (1xPBS, pH 7.4) and incubated in 5% normal goat serum/1xPBS/0.4% Triton X for 60min at room temperature. Rabbit polyclonal antibody (anti-Fos, Santa Cruz Biotechnology, sc-52) was utilized to recognize Fos in specific sections containing the dorsal hippocampus, amygdala, and medial prefrontal cortex. This antibody was used at a dilution of 1:2500 in 5% normal goat serum/1xPBS/0.4% Triton X. Following incubation (48h, 4 ^{0} C), sections were incubated with avidin-biotin-

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peroxidase complex (Vectastain ABC kit) for 45 min, then washed again in 1xPBS and processed using DAB with nickel-intensification (DAB peroxidase substrate kit, Vector Laboratories). Brain sections from each experimental group were processed similarly throughout all stages of the procedure. This procedure was adapted from Nikulina, et al. (2004) and used recently (Hoffman *et al.*, 2013).

Fos Protein Analysis

Tissue sections were examined for the presence of a blue-black reaction product indicating immobilized antigen. For each group, data were obtained from 2-6 sections/rat through each brain subregion in both hemispheres, and averaged to obtain a mean value. Selected areas (30,000 μm^2 for the hippocampal regions and 150,000 μm^2 for the mPFC and amygdala regions) were captured and digitized using a camera (CX9000, MicroBrightField, Burlington, VT) interfaced to a microscope (Olympus BX51) with a 20x objective. A profile was considered labeled if its pixel intensity was more than 2 standard deviations darker than the background, as calculated by Stereo Investigator software (MBF Biosciences). For hippocampal analyses, targeted subregions included CA1, CA3, and the suprapyramidal (or dorsal, upper) and infrapyramidal (or ventral, lower) blades of the dentate gyrus (DG_{sup} and DG_{inf}, respectively). Sampling regions within each subregion were identified consistently among each hippocampal slice (Fig. 10B). Once each subregion was identified at 20x, the subregion was outlined and Stereo Investigator calculated the area (mm^2). All positively labeled profiles were quantified and that value was divided by the area value to determine a density value. For the mPFC and amygdala analyses, adjacent cresyl violet stained sections were used to localize subregions with high confidence because these regions express poorly defined borders;

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for mPFC, analyzed subregions included anterior cingulate cortex (ACG), prelimbic cortex (PL), and infralimbic cortex (IL), for the amygdala, analyzed subregions included basolateral amygdala (BLA), central amygdala (CEA), and medial amygdala (MEA). Fos-IR labeling was quantified using a systematic random approach to achieve unbiased counts by an experimental blind to treatment conditions. Stereo Investigator software partitioned each image into 20 equal counting frames (100 x 75μm each), half of which were randomly selected and analyzed. The number of labeled Fos profiles was counted separately for each frame, excluding any overlapping labeled profiles on the left and bottom borders. Labeling density was calculated by dividing the estimated total number of labeled profiles by the total area analyzed. This procedure was adapted from Fanous et al. (2011) and reported recently .

Data Analysis

Data were analyzed by analysis of variance (ANOVA), and when significant effects were detected at p-value of 0.05 or less, post hoc analyses were performed. Additionally, planned comparisons were performed on data for which expected outcomes were anticipated based upon published findings. Proportions of freezing to CS were determined for associative memory during extinction 1 and 2 and were analyzed by one-sample *t*-tests with test statistic being 0.5. Data were analyzed by SPSS Version 19 and are represented as means ± SEM, with 8-10 animals/group for CON and STR and 4-5 animals/group for testing on the last day and Fos analyses.

Results

Stressor Effectiveness

Body weight gain and adrenal weight measures established chronic restraint as an effective stressor. A mixed factor ANOVA for restraint stress across weeks displayed a significant stress × week interaction $(F(3,54)= 72.19, p< 0.001)$. Whereas both STR and CON exhibited similar body weights at the start of the study (baseline), STR rats gained weight more slowly than did CON over the course of the three-week stress paradigm (Fig. 6B). A one-way ANOVA on stress history for comparing adrenal weight per 100 grams of body weight showed a significant main effect of restraint stress $(F(1,17)= 13.029, p< 0.01)$. The STR group showed significantly greater adrenal weights per 100 grams of body weight than the CON groups (Fig. 6C).

Fear Conditioning: Acquisition

Chronic stress facilitated fear acquisition during the last training trial. A mixed factors ANOVA for stress history across the three training trials revealed a significant effect of trial $(F(2,34)= 248.81, p< 0.001)$, demonstrating that both groups increased freezing across training trials. While no significant main effect for stress history or interaction between stress history and trial was found, past work has reported chronic stress to facilitate acquisition in fear conditioning at the second and third trials (Conrad *et al.*, 1999; Hoffman *et al.*, 2010). Consequently, planned comparisons were performed on trials 2 and 3 and revealed a significant effect of stress history on trial 3 $(F(1,17)=4.25, p= 0.05)$, with STR freezing more than did CON (Fig. 7A).

Fear Conditioning: Tone Extinction

While both groups exhibited decreased levels of freezing to tone across extinction blocks during Extinction 1, chronic stress slowed extinction learning. A mixed factors omnibus ANOVA for stress history across extinction blocks revealed a significant effect of block ($F(4,68)=18.08$, $p<0.001$) with no other significant effects. Given the findings reported by Izquierdo and colleagues (2006) that chronic stress slows the rate of extinction to tone, we performed planned comparisons during extinction; a one-way ANOVA for extinction block 3 revealed a significant effect of stress history ($F(1,17)=5.01$, $p<0.05$) with STR freezing more during to the CS during this block, midway through the session (Fig. 7B).

A history of chronic stress also caused robust recovery of freezing to tone 24h following the last trial of the first extinction session. A mixed factors ANOVA for stress history across the last block of the first extinction session and the first block of the second extinction session revealed a significant effect across blocks ($F(1,17)=26.19$, $p<0.001$), with both groups showing spontaneous recovery of fear to the CS, and a marginal stress x block interaction ($p=0.06$), suggesting that this increase may differ depending on stress history. A one-way ANOVA revealed a significant effect of stress for freezing to tone during the first block of Extinction 2, ($F(1,17)=8.58$, $p<0.001$), suggesting that chronic stress caused significantly greater recovery of fear responding to tone after extinction.

Both groups decreased freezing to tone during Extinction 2. A mixed factors ANOVA for stress history across blocks of extinction during Extinction 2 revealed a significant effect of block ($F(4,68)=29.40$, $p<0.001$). No other effects were significant.

Fear Conditioning: Context Extinction

For all rats, Extinction 1 and 2 occurred in the conditioning context, and freezing to the context was sampled during the 30s prior to tone onset for each trial during each extinction session. While both groups froze less to the context across the Extinction 1 session, chronic stress significantly impacted contextual fear as exhibited by greater levels of freezing prior to tone onset during Extinction 1. This was supported by a mixed factors ANOVA for stress history across context extinction blocks revealing a significant effect of block ($F(4,68)=27.03$, $p<0.001$), and a significant main effect of stress history ($F(1,17)=5.76$, $p<0.05$). There were no group differences during the first block of Extinction 2 for freezing to context. During Extinction 2, STR froze similarly to context as CON, although both groups froze less to the context as extinction blocks progressed, which was supported by a significant effect of trial, ($F(1,17)=18.85$, $p<0.001$), with no other significant effects (Fig. 7C).

Fear Conditioning: Proportion of freezing to CS during Extinction 1 and 2

To determine the relative contribution of freezing to the tone over the context for both CON and STR groups, proportions of freezing to CS were determined for Extinction 1 and 2. For both Extinction 1 and 2, freezing to either tone or context (30s prior to tone onset) was collapsed across all trials within a session and a proportion was computed as follows: the average number of seconds freezing during tone presentations divided by the sum, which was the average number of seconds freezing during tone presentations and the average number of seconds freezing to context just prior to tone onset [mean CS/ (mean CS + mean Context)]. A proportion of 1.0 indicates freezing to tone only without freezing to context (selective freezing to tone), whereas a proportion of 0.5 represents

equal freezing to both tone and context (nonassociative freezing). Proportions of freezing to CS for each group were analyzed by a one-sample t-test compared to the test statistic of 0.5 to evaluate selective freezing to the tone CS. During Extinction 1, only the CON group showed significant selective freezing to the CS (CON t(7)=3.901, p<0.01), whereas the STR group froze similarly to the CS and context (STR, p=0.27). During Extinction 2, both CON and STR groups showed selective freezing to the CS (CON, t(7)=3.273, p<0.05; STR t(9)=2.705, p<0.05) (Fig. 8). From these data, we conclude that the STR group exhibited non-selective freezing during Extinction 1, which contrasts to the CON group that demonstrated selective freezing to CS for both Extinction 1 and 2.

Fear Conditioning: Fear Generalization

Twenty-four hours following the second extinction session, both groups were subdivided and tested in either the same (SAME) or a novel chamber context (NOVEL), to assess fear generalization. Freezing behavior was analyzed prior to - (context) and during the first tone in the session. A 2 x 2 x 2 mixed factors ANOVA was performed for two between subjects factor for stress history (CON, STR) and test context (SAME, NOVEL) and one within subject factor for stimulus (freezing prior to - (context) and during the first CS trial). While there was a significant effect for stimulus (F(1,14)=35.805, p<.001), indicating that rats increased freezing during CS presentation compared to the context preceding the CS, we also found a significant stress x stimulus interaction, F(1,14)=8.595, p=0.01. Post hoc analyses revealed that chronically stressed rats showed high levels of freezing that barely changed between context (38.2 ± 10.6%) and CS (57.6 ± 12.0%), whereas CON showed significant increases in freezing from context (3.8 ± 1.6%) to CS (64.7 ± 11.8%, Fig. 9A). Specifically, chronically stressed rats

froze significantly more to the context prior to CS presentation than did the CON (t(16)=2.862, p=0.01) and the chronically stressed rats freezing to context increased during freezing to the CS (t(9)=2.686, p<0.05). In contrast, CON barely froze to context, and freezing robustly increased during CS presentation (t(7)=4.916, p<0.01, Fig. 9A).

Equally important, there was no significant contribution of test context for either stress condition (SAME, NOVEL). Consequently, STR froze more and similarly to context, regardless of whether they were tested within the same context or a novel context than did CON (further illustrated in Fig. 9B). Together, these data suggest generalization in the STR cohort. No other effects were significant from the omnibus ANOVA.

Fos IR labeling: Amygdala

Ninety minutes following the test in either the same or a novel context, all rats were perfused and brain tissue was processed for Fos immunohistochemistry. A two-way ANOVA for stress history and test context for Fos IR labeling within the basolateral (BLA) subregion revealed a significant main effect of stress history (F(1,15)=6.31, p<0.05), showing that regardless of test context, chronic stress increased Fos labeling during fear memory retrieval (Fig. 10A. The same analysis for the central amygdala (CEA) revealed a significant stress history x test context interaction (F(1,14)=4.36, p=0.05), with post hoc analyses showing STR-NOVEL had increased Fos IR labeling in the CEA vs. CON-NOVEL (p<0.001; Fig. 10A). There were no other significant effects for these subregions or for the MEA.

Fos IR labeling: mPFC

There was greater functional activation within the anterior cingulate (ACG) subregion of the mPFC when rats were tested in a novel context, regardless of stress history (Fig. 10A). This was supported by a two-way ANOVA for stress history and test context for Fos IR labeling within the anterior cingulate (ACG), revealing a significant main effect of test context ($F(1,14)=4.30$, $p=0.05$), no other effects were significant.

Fos IR labeling: Hippocampus

Within the hippocampal CA1 subregion, chronic stress enhanced functional activation during fear memory retrieval. A two-way ANOVA for stress history and test context for Fos IR labeling within the CA1 subregion revealed a significant main effect of stress history ($F(1,15)=5.102$, $p<0.05$), showing that regardless of test context, chronic stress increased Fos expression during fear memory retrieval. (Fig. 10A) There were no other significant effects.

Discussion

The current study aimed to address how a history of chronic stress affects fear extinction and nonassociative fear, and corresponding functional activation in brain regions sensitive to stress and involved with extinction. Here, we corroborate others' findings by demonstrating that a history of chronic stress impairs extinction learning (Izquierdo *et al.*, 2006) and memory (Miracle *et al.*, 2006; Baran *et al.*, 2009). Importantly, we extend these findings and are the first to show that chronic stress increases contextual fear during extinction, and fear generalization whether tested in a novel context. We also investigated patterns of functional activation using Fos IR labeling in brain areas sensitive to stress and fear extinction during an extinction recall

test in either the same or a novel context. We found that regardless of test context, chronically stressed rats showed increased Fos IR labeling in both the BLA and dorsal hippocampus CA1 region, compared to nonstressed controls. We also show that when tested in a novel context, chronic stress increased Fos expression in the CEA compared to nonstressed controls. Lastly, control or stressed rats that were tested in a novel context had more Fos IR labeling in the ACG compared to those tested in the same context. These data suggest that chronic stress increases the functional activation in limbic regions associated with fear extinction (amygdala and hippocampus). Therefore, chronic stress appears to affect nonassociative fear and alter fear extinction behavior and respective functional activation within limbic regions associated with fear extinction.

Compared to non-stressed controls, chronically stressed rats exhibited slower fear extinction within the first extinction session and robust spontaneous recovery at the beginning of subsequent extinction sessions. In addition, during the first extinction session, chronic stress produced enhanced contextual fear. While chronically stressed animals froze more to the CS and context during extinction 1 and 2 (Fig. 7A) and to the context in extinction 1 (Fig. 7B), we also showed that this group had a lower proportion of freezing to the CS during extinction 1 (Fig. 8), demonstrating nearly equal freezing to the CS and context. Moreover, STR also demonstrated more freezing to the context when tested in a novel context, suggesting fear generalization (Fig. 9). These behavioral effects during CS extinction mimic the challenges faced with treating PTSD patients who are resistant to exposure-based therapy (Pitman *et al.*, 1996; Craske *et al.*, 2008). Additionally, the effects on nonassociative fear (sensitization, generalization) are relevant to the cluster of hyperarousal symptoms seen in PTSD patients (Yehuda & LeDoux,

2007). Taken together, the current study supports the use of chronic stress and fear conditioning as a familiar paradigm that can be implemented in novel ways to investigate an animal model for PTSD, with face validity for a PTSD-like behavioral phenotype.

Fos expression during post-extinction fear memory retrieval in the same and in a novel context revealed interesting patterns within subregions of the amygdala and dorsal hippocampus. We found that regardless of test context (SAME, NOVEL), chronically stressed animals had greater Fos IR labeling in the BLA and hippocampal CA1 region. These parallel patterns of activation are noteworthy, given the reciprocal connections between the amygdala and CA1 region of the hippocampus (Pitkanen *et al.*, 2000), and supports the recent findings that amygdala-hippocampal functional connectivity is enhanced following chronic stress (Ghosh *et al.*, 2013). As test context had no impact on behavior for our chronically stressed groups, the Fos effects within BLA and CA1 tended to reflect the behavioral patterns that we observed with generalization, however within the CEA we observed greater Fos expression in the chronically stressed group compared to controls when these groups were tested in a novel context. This activation pattern may be due to greater contextual freezing in the STR-NOVEL group, and may suggest a role for the CEA in contextual fear, as this group showed generalization to a novel context, while there was virtually no freezing to context in the CON-NOVEL group. We also found that regardless of stress history (CON and STR), rats were tested in a novel context had greater functional activation in the anterior cingulate cortex (ACG). These results are consistent with previous work that has indicated a role for the ACG to be involved in discriminating stimuli in fear conditioning paradigms (Powell *et al.*, 1994; Morgan & LeDoux, 1995). In the current paradigm, we did not observe differences in the IL or PL

of the mPFC, as found in another study with a similar paradigm (Knapska & Maren, 2009), however subtle differences such as an additional extinction session as in the current study may reflect the disparate findings, as Fos is known to habituate to novelty (Papa *et al.*, 1993). More research is needed to tease apart chronic stress effects on the contribution of associative to nonassociative fear responding in patterns of relevant brain activation.

A history of chronic stress resulted in a neurobiological and behavioral vulnerability to develop exaggerated fear responses during fear conditioning and extinction, and may be considered as an environmental risk factor for the development of PTSD following exposure to a traumatic event. As discussed earlier, chronic stress creates a structural imbalance of brain morphology in regions associated with fear processing, favoring the amygdala with deficits in regions involved in emotional regulation (mPFC), which may be mediating the persistent and generalized fear responding. In the current study, we are the first to show increased functional activation (via Fos IR labeling) within amygdala subregions (BLA and CEA) following chronic stress during recall of a fear memory. This parallels human functional imaging data that show greater amygdala activation in human populations with PTSD (Liberzon *et al.*, 1999; Shin *et al.*, 2006). Moreover, it has been observed that there is a reduction in hippocampal volume in PTSD patients (Bremner *et al.*, 1995; Woon *et al.*, 2010), but despite this, many functional imaging studies have reported greater hippocampal activation in this patient population (Sachinvala *et al.*, 2000; Osuch *et al.*, 2001; Shin *et al.*, 2006; Thomaes *et al.*, 2009; Werner *et al.*, 2009). These outcomes observed in humans with PTSD parallel what many have observed following chronic stress

considering dendritic atrophy in the hippocampus as an indirect measure of volume (Watanabe *et al.*, 1992; Tata & Anderson, 2010; Hoffman *et al.*, 2011). Furthermore, here we are the first to show greater Fos expression in the hippocampus following chronic stress during retrieval of a fear memory. Thus, this is the first study to show both behavioral and functional neurobiological parallels in an animal model of PTSD. Chronic stress induced alterations in limbic regions implicated in PTSD suggest that the amygdala and hippocampal functional network is disrupted and may underlie exaggerated fear and impairments in context discrimination.

CHAPTER 4

CHRONIC STRESS ENHANCED FEAR MEMORIES ARE ASSOCIATED WITH
INCREASED AMYGDALA *ZIF268* MRNA EXPRESSION AND ARE RESISTANT
TO RECONSOLIDATION IN AN ANIMAL MODEL OF POST TRAUMATIC
STRESS DISORDER

Chronic stress may impose a vulnerability to develop psychiatric conditions, especially anxiety disorders such as post traumatic stress disorder (PTSD) following a traumatic event. The clinical literature suggests that a history of trauma or stress prior to exposure to a traumatic event increases the prevalence of PTSD (Breslau *et al.*, 1999). Many structural and physiological alterations within the PTSD human brain (Miller & McEwen, 2006) correspond with changes observed in the rodent brain following chronic stress (McLaughlin *et al.*, 2009), including amygdala dendritic hypertrophy (Vyas *et al.*, 2002) and hyperexcitability (Rosenkranz *et al.*, 2010), as well as dendritic retraction in the medial prefrontal cortex (Izquierdo *et al.*, 2006) and hippocampus (Conrad *et al.*, 1999; Hoffman *et al.*, 2011). The similarity of these systems within the stressed brain suggests overlapping dysregulated mechanisms that require investigation not only for a better understanding of the PTSD brain, but also to elucidate potential effective therapeutic strategies.

Exploring chronic stress effects on fear conditioning behaviors may provide insight into the neural mechanisms associated with treatment resistant PTSD symptoms, as in this model, we observe a PTSD-like phenotype for traumatic memory. Specifically, chronic stress prior to Pavlovian fear conditioning results in facilitation of the acquisition (Conrad *et al.*, 1999; Hoffman *et al.*, 2010), resistance to extinction (Izquierdo *et al.*,

2006), increased contextual and generalized fear (Hoffman *et al.*, under review), and substantial increases in recovery of fear after extinction (Miracle *et al.*, 2006; Baran *et al.*, 2009; Hoffman *et al.*, under review) compared to animals without a history of chronic stress. Current treatments for PTSD that use exposure-based cognitive therapy focus on the facilitation of extinction learning, but challenges arise when fear responses occur between and outside the context of therapy sessions (discussed in Hartley & Phelps, 2010), which are commonly observed in traditional the literature for post-extinction effects (Bouton, 2004; Rescorla, 2004). Therefore, exploring novel treatment strategies to attenuate persistent fear memories, as we observe following chronic stress and fear conditioning, warrants further investigation.

One approach that has gained enthusiasm over the last decade is to target the original memory trace, by manipulating reconsolidation. Reconsolidation refers to the process of a previously stable, consolidated memory being retrieved or "reactivated" into an active state that renders the previously stored memory to be labile and vulnerable to change or disruption, a process that depends on protein synthesis (Przybyslawski & Sara, 1997; Nader *et al.*, 2000). Many studies show that administration of a variety of amnestic agents when a memory is reactivated can lead to amnesia for the original memory trace (reviewed in Tronson & Taylor, 2007), and may provide a novel approach for attenuating fear memories in PTSD**.**

One of the key targets in the reconsolidation process for weakening a fear memory is blocking protein synthesis within the reconsolidation window after memory reactivation (Nader *et al.*, 2000; Tronson & Taylor, 2007). The mammalian target of rapamycin (mTOR) kinase regulates a subset of protein synthesis in neurons at the level

of translation from the message to the protein (Hoeffer & Klann, 2010). Emerging evidence demonstrates that mTOR is involved in synaptic plasticity, as inhibition of the mTOR pathway by rapamycin, blocks consolidation and reconsolidation of a fear memory when applied directly to the amygdala (Parsons *et al.*, 2006) or administered systemically (Blundell *et al.*, 2008). Inhibiting mTOR via rapamycin following reactivation of a fear memory might be a novel approach in attenuating chronic stress enhanced fear memories.

We hypothesized that chronic stress leads to a vulnerability to develop enhanced maladaptive fear memory following a traumatic event through functional alterations in fear neurocircuitry, which may be vulnerable to disruption. We tested this hypothesis in a series of behavioral and histochemical experiments using a well-established chronic restraint stress model followed by Pavlovian fear conditioning and reconsolidation manipulations and *in situ* hybridization histochemistry. Post reactivation rapamycin was administered systemically to determine its effects on chronic stress enhanced fear memory within the time window that reconsolidation should occur. In conjunction with behavioral measures, we also asked whether chronic stress affects the relative functional activation of amygdala and hippocampal subregions during fear memory tests. Functional activation was measured using *in situ* hybridization histochemistry for the immediate early gene (IEG), *zif268* (also known as *egr-1, ngfi-a,* and *krox-24)* mRNA, induced during fear memory reactivation and post reactivation long term memory (PR-LTM). IEGs are one of the first genes expressed in response to a wide variety of stimuli, with *zif268* implicated in plasticity and reconsolidation (Bozon *et al.*, 2003; Maddox *et al.*, 2011). Identifying changes in stress- and fear-sensitive brain regions, such as the

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amygdala and hippocampus, during retrieval of an emotional memory can help us better understand chronic stress influences on these networks during retrieval of a fear memory.

Method

Subjects

One hundred-four male Sprague-Dawley rats weighing approximately 250-275g upon arrival (Charles River Laboratories) were pair housed in light and sound-attenuating chambers (21-22°C) on a 12:12 reverse light cycle (lights off at 6am) according to conditions specified by the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources on Life Science, National Research Council, 1996). Food and water was available *ad libitum* except during restraint procedure (described below). All procedures occurred during the dark phase of the light cycle.

Stress Manipulation

Rats were chronically stressed via repeated wire mesh restraint (STR) or not (CON), using procedures that have been carefully documented to produce the morphological and behavioral changes reflecting chronic stress (cite McLaughlin et al., 2007). During the designated restraint period, rats were restrained in their home cages in wire mesh restrainers for 6h/d/21d. Wire mesh restrainers were 18cm circumference x 24cm long (wire mesh from Flynn and Enslow Inc, San Francisco, CA) with wire ends sealed with grip guard sealer (ACE Hardware). Nonstressed controls (CON) were handled briefly each day, with food and water restricted during the duration of the 6h restraint for the STR rats to ensure similar levels of food and water access and handling by the experimenter.). Rats were weighed weekly.

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Fear Conditioning: Apparatus

Rodent fear conditioning chambers (25 cm depth x 29 cm height x 26 cm width: Coulbourn Instruments, E10-18TC) were contained in sound-attenuating cubicles (Coulbourn, E10-23, white). A PC interface card (Coulbourn, PCI-3-KIT) adapted to a PC, a universal link (Coulbourn, L91-04S), and Graphic State software (v 3.03 GS3.03) controlled the stimulus presentation. A frequency generator (Coulbourn, E12-01) produced a tone (75 dB, ~3.0 kHz) through a speaker located in the side panel of the conditioning chamber. The shock (500 ms, 0.25mA, Coulbourn Animal Shock Generator, H13-15) was administered as a current, equally distributed through a metal grid floor (Coulbourn, E10-18RF). Behavior was videotaped for off-line analysis using a camera (Coulbourn, E27-91) mounted on the ceiling and a videocassette recorder. Infrared lights, undetectable to the rats, were located on the side panels of the chamber to denote the onset and offset of the tone, since there was no audio on the videotaped recordings. A house light (Coulbourn, E11-01) was mounted in the side panel to illuminate the chamber.

Two distinct chamber contexts (contexts A and B) were utilized for different fear conditioning testing phases. Context A consisted of white and silver paneled walls, a wire bar shock floor with a white catch pan, and was cleaned with 70% ethanol. Context B consisted of striped paneled walls, a smooth Plexiglas® floor insert and a dark catch pan, and was cleaned with an orange scented cleaner (method® clementine all-purpose natural surface cleaner, methodhome.com).

Fear Conditioning: Procedure

In order to maximize the explicit association between tone (CS) and footshock (US), we adapted an acclimation paradigm to involve extensive context pre-exposure. Our past work revealed that minimal (1 session) context pre-exposure leads to nonspecific freezing to the context and CS and generalized fear to novel contexts in chronically stressed rats (Hoffman *et al.*, under review). Consequently, all testing groups were acclimated to both chamber contexts during the last six days of restraint stress. Rats were transported by cart in their home cage into the testing room and placed in each context A and B for 10min on alternating days (3 days each). The following day, all groups were placed into the conditioning chamber (context A) and after 140s, were subjected to three-30s tones that co-terminated with a .25mA, 500ms shock (variable ITI 180-360s). After the end of the conditioning session, all rats were transported back to their home colony.

One day after conditioning, rats were placed in context B and presented with one 30s CS reactivation trial. Immediately after, rats were injected with either rapamycin (RAPA; 40 mg/kg, i.p.) at a dose that blocks reconsolidation of a fear memory when administered systemically (RAPA; 40mg/kg, i.p., Blundell *et al.*, 2008) or an equivalent volume of vehicle (VEH). After injections, all groups were returned to their home colony.

Four and 24h following CS reactivation and injections, all rats were tested for post-reactivation short term memory (PR-STM) to determine whether RAPA affects amygdala-dependent freezing behavior. Twenty hours later, all groups were tested for PR-LTM to determine RAPA effects on reconsolidation of a long term fear memory. For each test, rats were transported in their home cages and placed in the same chamber as

the CS reactivation (context B) and received 3-30s tone presentations, then were

transported back to the colony.

To determine the long lasting effects of chronic stress and post-reactivation

RAPA on fear memory, one week after PR-LTM, all groups were tested for spontaneous

recovery by placing them in context B and presenting them with 3-30s CS (as in PR-STM

and PR-LTM).

Footshock detection threshold

Two days after testing for spontaneous recovery, rats were tested for footshock

detection threshold to determine whether a history of stress or systemic RAPA altered the

threshold to detect a footshock. Briefly, rats were placed in the fear conditioning chamber

and presented with unsignaled footshocks that increased in intensity by 0.05mA

increments until two different responses are reached: notice (slight orienting head

movement), and jump (paws briefly raised off the chamber floor, Conrad *et al.*, 1999;

Baran *et al.*, 2009; Baran *et al.*, 2010; Hoffman *et al.*, 2010). After a jump response was

observed, the footshock at the same amperage was presented again, and the footshock

intensity decreased until jump and notice responses were no longer observed. The two

measures for notice and jump that determined when the amperage increased and

decreased were averaged to represent one value in the data analyses. A general

experimental timeline is illustrated in Fig. 11.

Drug preparation and administration

Solutions of RAPA and VEH were made with 1% DMSO and 5% Tween-80 in

sterile saline two days before use. Briefly, the vehicle solution was mixed and RAPA was

added (or not for VEH) and sonicated until mixed into suspension. The dose for RAPA

was 40mg/kg based on previously published work demonstrating effective blockade of reconsolidation of a contextual fear memory when administered systemically (Blundell *et al.*, 2008). The solutions were refrigerated overnight. On the day of use, the drug was vortexed prior to each i.p. injection to ensure thorough mixing.

Fear Conditioning: Dependent Variable

Behavior was videotaped for scoring later by observers blind to experimental conditions. The dependent variable measured was defined as the number of seconds freezing during each 30s tone presentation (freezing to CS) or the number of seconds freezing during the 30s interval prior to CS presentation (freezing to context). Freezing was defined as the absence of all movement except those associated with respiration (Blanchard & Blanchard, 1969; Conrad *et al.*, 1999; Quirk *et al.*, 2000; Baran *et al.*, 2009; Baran *et al.*, 2010; Hoffman *et al.*, 2010) and represented as a percentage of the 30s interval.

zif268 *mRNA* in situ *hybridization tissue preparation*

A subset of rats (n=16/group) that were either chronically stressed (STR) or not (CON) were euthanized after the reactivation trial and brains were processed for *zif268 in situ* hybridization histochemistry for mRNA expression. Half of each group served as a conditioning naïve control and were not subjected to the US (footshock) or CS in the context chambers (naïve) to determine the effects of chronic stress and conditioning on retrieval-induced *zif268* mRNA expression.

Another subset of rats (n=16/group) were tested through PR-LTM and euthanized for brains to be processed for *zif268 in situ* hybridization histochemistry for mRNA expression. For these cohorts of rats, they were euthanized one hour following fear

memory reactivation and/or PR-LTM to capture peak expression of *zif268* mRNA. Rats received an injection of Euthasol (100mg/kg, i.p.), and then were transcardially perfused with cold phosphate buffered saline (PBS) followed by 4% paraformaldehyde in PBS. Brains were removed and post fixed in 4% paraformaldehyde for approximately 24h and were cryoprotected in 15% and 30% sucrose solutions over 2d. Coronal sections were taken at 20μm using a cryostat, mounted onto charged slides (Fisherbrand™ Superfrost™ Plus, Thermo Fisher Scientific, Inc.) and stored at -80°C until further processing.

Selected slides were hydrated in two rinses of buffer solution and acetylated in 0.25% acetic anhydride in 0.9% NaCl/0.1M triethanolamine (TEA) for 10min, then dehydrated for 1min in a graded series of ethanol washes as follows: 50, 75, and 95% in diethylpyrocarbonate-treated (DEPC) water and 100% ethanol. Slides were then delipidated in chloroform for 5min, washed for 1min in 100% ethanol and air dried for approximately 20min. All slides were then processed at the same time using the same batch of hybridization solution to ensure a homogenous concentration of probe on all sections.

Probe Labeling

An oligodeoxyribonucleotide probe sequence complementary to amino acids 2-16 of the *zif268* protein (5'CCGTTGCTGAGCATCATCTCCTCCAGTTTGGGGTAGTT GTCC3') was 3' end labeled with [^{35}S]dATP (Perkin Elmer Analytical and Life Sciences) utilizing terminal deoxynucleotidyl transferase (Gibco BRL). The procedure for labeling has been described previously (Hammer & Cooke, 1996; Covington *et al.*, 2005). Briefly, the probe was purified and diluted with hybridization buffer containing 50% formamide, 500μg ml^{-1} sheared salmon sperm DNA, 250μg ml^{-1} yeast tRNA, 4x

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saline-sodium citrate (SSC), 1x Denhardt's solution, and 10% dextran sulfate combined with DTT (2μl 5M DTT/100μl solution) in order to yield a 3×10^7 cpm/ml hybridization solution.

In situ *hybridization histochemistry*

Approximately 100μl of the hybridization solution was added to each slide under sterile coverslips for approximately 16h at 37°C. Coverslips were removed and slides were rinsed in 1xSSC at 23°C. Slides were then washed four times: 2xSSC for 1h, 1xSSC for 1h, 0.5xSSC for 30min, and 0.1xSSC at 37°C for 30min and then rinsed in 0.1xSSC, washed twice in 1xSSC for 30min and dehydrated again in a series of graded ethanol before being air dried for 30min. Dried slides were exposed to Biomax-MR for 6d and developed with D19 and Rapid Fixer (Kodak).

Image analysis

Regional autoradiographs were assessed in defined brain regions by digitizing them using a CCD camera connected to a Macintosh computer. Neuronal *zif268* mRNA expression was measured in lateral, basal, central (central/lateral and medial divisions) and medial nuclei (LA, BA, CeL/C, CeM, and MeA, respectively), hippocampal CA1, CA3, and suprapyramidal blade of the dentate gyrus (DG). Following film development, the same processed slides were counterstained with Nissl to determine amygdala subregion anatomy. Film images were aligned with Nissl images and calibrated optical density was measured. Regions of the hippocampus (CA1, CA3, and DG) were assessed using a manual outline of each region. Quantification of optical density in all sections were analyzed with image-analysis software using calibrated radiostandards (ImageJ) to

generate a ^{35}S calibration curve in μCi/g. Data from the right and left hemispheres were combined to generate a mean value for each selected brain region.

Data Analysis

Separate cohorts of animals were tested at different times, and when treatments were identical (i.e., prior to *in situ* hybridization analysis), behavioral data were combined and described in the results. Cohort was treated as an independent variable in the analyses to rule out any cohort x treatment interactions. Data were analyzed by analysis of variance (ANOVA), and when significant interactions were detected at a p-value of 0.05 or less, post hoc t-test analyses were performed. Planned comparisons were performed by t-test where indicated.

Results

Effects of chronic stress on fear conditioning and memory reactivation.

Chronic stress facilitated fear learning and enhanced freezing during memory reactivation. Three cohorts of rats that were either chronically stressed (STR) or not (CON) were fear conditioned with three CS-US trials in context A as illustrated in Fig. 12A. A mixed factors ANOVA for cohort and stress across the three conditioning trials revealed a significant effect of conditioning trials, $F(2, 164)=466.8$, $p<0.001$, whereby rats in all groups froze more as trials progressed. A significant effect of stress, $F(1, 82)=6.379$, $p<0.05$, $\eta^2=0.072$, and a significant stress x conditioning trial interaction, $F(2, 164)=3.491$, $p<0.05$, $\eta^2=0.096$, was also found. Post hoc analyses indicated that the chronically stressed groups froze more to the CS during trials 2 ($t(86)=2.61$, $p<0.05$) and 3 ($t(86)=1.971$, $p=0.05$) compared to nonstressed controls (Fig. 12B). No other effects were significant and testing cohort failed to interact with any variable.

The following day, all groups were tested for memory reactivation (freezing to 1 CS trial) in a different context (context B, Fig. 12A). A 3x2 ANOVA for cohort and stress for freezing to tone during reactivation revealed a significant effect of stress, $F(1, 82)=16.836$, $p<0.001$, $\eta^2=0.17$, with no other significant effects. Chronically stressed rats froze more to the CS than did the controls during reactivation, with chronically stressed rats freezing at 95.1% and controls freezing at 83.2% (Fig. 12C). These effects were not due to nonassociative fear, as freezing to context prior to the reactivation trial was measured in a subset of rats representing both groups (cohort 1, CON-CSUS vs. STR-CSUS) did not reveal a significant effect for freezing to context $t(14)=.373$, NS, Fig. 12D. No other effects were significant.

Effects of chronic stress and fear conditioning on amygdala and hippocampal zif268 *mRNA during fear memory reactivation.*

One hour following the reactivation session, a subset of chronic stress and control groups were euthanized following the one reactivation trial (CON-CSUS, STR-CSUS) and were matched with naïve groups that were placed in the fear chambers for the same amount of time without US or CS presentation (CON-naïve, STR-naïve, Fig. 13A). Five subregions within the amygdala and three hippocampal subregions were analyzed for μCi/g by ImageJ following *zif268 in situ* hybridization. Two-way ANOVAs for stress (CON, STR) and conditioning condition (CSUS, naïve) on μCi/g for each amygdala and hippocampal subregion were performed. Analyses revealed a significant main effect of conditioning for the LA $(F(1, 28)=39.244, p<0.001)$, and a marginal stress x conditioning interaction $(F(1,28)=3.525, p=0.07)$. The conditioning effect demonstrates that groups that were fear conditioned (CSUS) expressed more LA *zif268* mRNA during reactivation

than did naïve groups (Fig. 13B), and relative expression may depend on chronic stress prior to conditioning. Similar effects were observed within the BA, where we found a significant main effect of conditioning (F1,28)=12.10, p<0.005 and a significant stress x conditioning interaction (F(1,28)=13.065, p<0.001), indicating that fear conditioning-induced *zif268* mRNA within the BA differs depending on prior stress. Post hoc analyses showed that the chronically stressed fear conditioned group (STR-CSUS) had greater *zif268* mRNA expression compared to fear conditioned controls (CON-CSUS; t(14)=2.357, p<0.05). Further, there was a significant difference in *zif268* mRNA expression for naïve groups, with CON-naïve having greater *zif268* mRNA levels than STR-naïve (t(14)=2.905, p<0.05). We were also interested in the effects of chronic stress on relative *zif268* mRNA expression during fear memory reactivation, therefore planned comparisons for CON-CSUS vs. STR-CSUS groups were analyzed by t-tests for each subregion analyzed. These results revealed significant increases in *zif268* mRNA expression in the STR-CSUS group within the LA (t(14)=2.507, p<0.05), and in the BA (stated above) compared to CON-CSUS (Fig. 13, ^p<0.05 vs. CON-CSUS). A breakdown of all brain regions sampled is detailed in Table 1. No other effects were significant.

Rapamycin does not impact post-reactivation short-term memory (PR-STM)

Two cohorts of chronically stressed and nonstressed controls were fear conditioned in context A then 24h later the memory was reactivated with one CS presentation in context B. Immediately following reactivation, all groups were injected with either rapamycin (RAPA, 40mg/kg, i.p.) or equivalent volume of vehicle (VEH). Four hours later, all groups were tested for PR-STM (Fig. 14A). Regardless of drug

condition, chronically stressed rats froze more to the CS during PR-STM, as revealed by a 2x2x2 ANOVA for cohort, drug, and stress with a significant main effect of stress, $F(1,64)=3.714$, $p=0.05$, $\eta^2=0.055$ (Fig. 14B). No other effects were significant.

Chronic stress enhanced fear memories were resistant to reconsolidation

For the PR-LTM assessment 20 hours following PR-STM, rats were placed back into context B and presented with 3x CS trials (Fig. 15A). A 2x2x2 ANOVA for cohort, drug condition, and stress revealed a significant main effect of cohort, $F(1, 64)=10.513$, $p<.0.01$, $\eta^2=0.141$. This effect reflects a general increase in freezing to tone for all groups in cohort 3 compared to cohort 2. A lack of significant interactions was found, suggesting that any observed significant experimental effects were unaffected by experimental session and were thus represented as combined (Fig. 15B). There was also a significant main effect of stress, $F(1, 64)=5.337$, $p<0.05$, $\eta^2=0.077$; consistent with the observation at PR-STM, where chronically stress groups froze more to the CS than did nonstressed controls. Additionally, we observed a significant main effect of drug, $F(1, 64)=13.039$, $p=0.001$, $\eta^2=0.169$ whereby groups that received RAPA following CS reactivation showed a decrease in freezing to tone during PR-LTM than did VEH groups (Fig. 15B). Since we were interested in looking further into chronic stress effects of post reactivation rapamycin on PR-LTM, we ran planned comparisons for CON and STR alone. For nonstressed controls, a 2x2 ANOVA for cohort and drug on freezing to tone CS during PR-LTM revealed a significant main effect of cohort, $F(1,32)=7.308$, $p<0.05$ (consistent with the omnibus ANOVA effects), and a significant main effect of drug, $F(1,32)=11.066$, $p<0.01$, indicating that post reactivation rapamycin significantly attenuated freezing to tone during PR-LTM in nonstressed controls (Fig. 15B).

Interestingly, when we probed the rapamycin effect for PR-LTM for groups that were chronically stressed, the drug effect was eliminated, as revealed by nonsignificant effects of drug F(1,32)=3.214, p=0.082. No other effects were significant, including cohort F(1,32)=3.586, p=0.068, suggesting that regardless of cohort or drug condition, chronically stressed groups froze similarly to the CS during PR-LTM (Fig. 15B).

Effects of chronic stress and post reactivation rapamycin on amygdala and hippocampal zif268 *mRNA during PR-LTM.*

One hour after PR-LTM test for all groups, a subset of animals (n=8/group) were euthanized and brains were processed for *zif268 in situ* hybridization (Fig. 16A). The same subregions with in the amygdala and hippocampus were analyzed as in CSUS and naïve groups described previously. Two-way ANOVAs for stress (CON, STR) and drug condition (VEH, RAPA) on µCi/g for each amygdala and hippocampal subregion were performed. Analyses revealed significant main effects of stress for amygdala LA ($F_{(1,22)}=8.967$, *p*<0.01), BA (F(1,26)=4.496, *p*<0.05), and hippocampal CA1 (F(1,27)=6.725, *p*<0.05) regions. Regardless of drug condition, chronically stressed groups expressed significantly more *zif268* mRNA within the amygdala subregions (LA, Fig. 16B, **p<0.01; BA, Fig. 16C, *p<0.05), and in contrast, significantly less *zif268* mRNA expression within CA1 of the hippocampus (Fig. 16D, *p<0.05) compared to nonstressed controls. No other effects were significant. A breakdown of all brain regions sampled is detailed in Table 2. Representative photomicrographs for the *zif268 in situ* hybridization at PR-LTM for the LA, BA, and CA1 stained sections are illustrated in Fig. 17.

Effective post reactivation rapamycin effects are long lasting in attenuating a long term fear memory

In the remaining cohort of animals that were not used for *zif268* mRNA analyses, we examined additional behaviors including spontaneous recovery, where all groups were tested for freezing to CS in context B one week following PR-LTM, Fig. 17A. A 2x2 ANOVA for stress and drug revealed a significant stress x drug interaction, $F(1,36)=4.385$, $p<0.05$, indicating that the lasting effects of rapamycin on long term fear memories depends on whether the groups were previously chronically stressed. Post hoc analyses revealed that nonstressed controls that received post reactivation rapamycin (CON-RAPA) displayed significantly lower levels of freezing to the CS during spontaneous recovery than those that received vehicle (CON-VEH; $t(18)=3.19$, $p<.01$), while RAPA did not affect freezing in chronically stressed animals, $t(18)=.634$, NS, Fig. 18B.

It was also important to demonstrate whether the behavioral effects of chronic stress and rapamycin were associative, and not due to nonassociative effects or generalized freezing one week later. Consequently, freezing to context was measured by quantify freezing during the 30s interval prior to each CS trial. A 2x2 ANOVA for stress and drug on freezing to context B during spontaneous recovery revealed a significant main effect of stress ($F(1,36)=3.933$, $p=0.05$), with chronically stressed rats freezing about 7.6% of the time compared to controls freezing about 15.9%, Fig. 18C. To represent the amount of freezing to CS compared to context, the data were transformed into a proportion of freezing to the CS during this session. The proportion was calculated as freezing to (CS/CS+context), where a proportion of 1 indicates selective freezing to

the tone, and a proportion of 0.5 as freezing equally to the tone and context

(nonassociative freezing). These data were averaged across the three trials and analyzed

for each group by a one-sample t-test against test statistic of 0.5 to show selective

freezing to the CS. All groups showed significant selective freezing to the CS (CON-

VEH, $t(9)=11.629$, $p<0.001$; CON-RAPA, $t(9)=14.141$, $p<0.001$; STR-VEH, $t(9)=9.97$,

$p<0.001$; STR-RAPA, $t(9)=11.231$, $p<0.001$, Fig. 18D). These data strongly support

associative learning occurred. Consequently, chronic stress may slightly increase freezing

to the test context, but the significant and selective freezing to CS indicates that all

groups showed greater selectivity of freezing to tone.

Rapamycin increases footshock detection threshold

To determine whether stress or drug treatment affected footshock detection

threshold, footshock intensity was slowly increased at 0.05 mA to determine each rats'

orienting and jump response. The threshold for notice/orienting was unaffected by stress

or rapamycin with a mean range from (0.029mA to 0.031mA, Fig. 19). In contrast,

groups that were administered post reactivation rapamycin (RAPA) tended to have a

higher footshock threshold for a jump response than did those treated with VEH,

$F(1,36)=9.943$, $p<0.01$, (Fig. 19, *$p<0.01$ vs. VEH). No other effects were significant.

Chronic restraint attenuates body weight gain.

For all cohorts of rats, body weight was recorded weekly throughout restraint

duration to determine stressor effectiveness. A mixed factors ANOVA for cohort, stress,

and weeks across chronic restraint on body weight revealed a significant effect of weeks

($F(3,294)=2243.098$, $p<0.001$), a significant cohort x weeks interaction ($F(6,294)=9.435$,

$p<0.001$), a significant weeks x stress interaction ($F(3,294)=218.691$, $p<0.001$), and a

significant weeks x cohort x stress 3-way interaction ($F_{(6,294)}=2.218$, $p<0.05$). Post hoc tests for stress effects on body weight within each week demonstrate that all groups weighed similarly on day 1 ($t_{(102)}=.74$, NS), but chronic stress groups weighed significantly less during the subsequent weeks (day 7, $t_{(102)}=10.807$, $p<0.001$; day 14, $t_{(102)}=11.308$, $p<0.001$; day 21, $t_{(102)}=12.044$, $p<0.001$). Although rats were ordered of the same weight, there was some variation across cohorts. However, the pattern of chronic stress altering body weight gain was consistent in which chronic restraint stress reliably attenuated body weight gain across the three week restraint duration, Fig. 20. A breakdown of weights for each cohort across weeks is defined in Table 2.

Discussion

Chronic stress is well documented to enhance fear memories and these data are the first to demonstrate that these chronic stress enhanced fear memories are resistant to reconsolidation disruption via rapamycin through mechanisms that likely involve enhanced amygdala activation. Our findings demonstrate that chronic stress enhanced freezing during fear memory reactivation was associated with augmented amygdala (LA and BA) *zif268* mRNA induction, this enhancement persisted at a later memory retrieval test (PR-LTM) when hippocampal functional activation was decreased. Moreover, rapamycin's effects to disrupt reconsolidation in the non-stressed controls was unlikely due to neural damage as all groups demonstrated intact fear memory when tested for short term memory that is independent of protein synthesis. Consequently, systemic administration of rapamycin following post-reactivation was verified to be effective at attenuating fear memory, as measured by reduced freezing during post reactivation long term memory tests (PR-LTM and spontaneous recovery) in nonstressed controls.

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However, this reduction in fear responding was not observed in our chronically stressed groups with a PTSD-like phenotype. These results demonstrate a potential challenge in the application for reconsolidation manipulations in the treatment of strong fear memories in PTSD populations.

A unique feature of this study is the careful attempt to minimize variables that would influence nonspecific freezing in an auditory fear conditioning paradigm, which is particularly important to consider in chronic stress and fear conditioning paradigms. We have previously shown that chronic stress can potentiate contextual fear and enhance generalization to novel contexts (Hoffman *et al.*, under review), which resembles a PTSD-like phenotype but can cloud interpretations for associative learning. In the current studies, it was important to create an explicit association between CS and US, with minimal influence of the context with the intent to disrupt an associative fear memory. Therefore, we adapted a thorough context pre-exposure paradigm, in which all groups were acclimated to both test chamber contexts 3x each over the 6d prior to conditioning to ensure habituation to the chambers and minimize the impact of context during CS-US conditioning. Furthermore, given that we sought to target a specific associative memory, we preformed the reactivation and test sessions in a context distinct from the conditioning context, which has shown to be important in memory reconsolidation induction (Finnie & Nader, 2012). With these measures, we demonstrated selective responding to the CS over the context for both control and chronically stressed groups, as we show under these parameters very low levels of freezing to the context both during fear memory reactivation (Fig. 12C) and spontaneous recovery (Fig. 18C). Without such procedural care, the chronically stressed rats could have exhibited nonspecific fear generalization as

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shown previously (Hoffman *et al.*, under review). Although we saw that the chronic

stress groups in the current paradigm showed significantly greater freezing to context

prior to tone onset during spontaneous recovery, these levels were low (<16%) and the

proportion of freezing to the CS demonstrated significant selective freezing to the cue.

These data in conjunction with the rapamycin effects support that associative learning

occurred, and that post reactivation rapamycin treatment effectively blocked

reconsolidation of an auditory fear memory in nonstressed controls that was long lasting,

while failing to do the same in the chronically stressed rats

As found in other reports (Conrad *et al.*, 1999; Baran *et al.*, 2009), chronic stress

had no impact on the footshock threshold to detect a notice or jump response. However,

rapamycin treatment appeared to significantly increase the threshold to detect footshock

for a jump response, although there was no effect at the notice response. This outcome

was unlikely to impact the representation of the footshock itself (but see Rescorla, 1994),

as the injection of rapamycin was administered the day after the presentation of

footshocks during conditioning. Moreover, no freezing differences were observed for

rapamycin injection at the PR-STM timepoint. Upon a literature review, rapamycin may

play a role in reducing pain sensitivity (Weragoda & Walters, 2007; Geranton *et al.*,

2009; Lisi *et al.*, 2012). However, not all studies have found rapamycin to alter footshock

detection threshold (Blundell *et al.*, 2008). Effects of rapamycin on pain sensitivity occur

when an organism has previously been exposed to noxious stimuli, such as footshock

(fear conditioning, current study), formalin (Price *et al.*, 2007), or capsaicin (Geranton et

al., 2009), but not when assessed in naïve conditions. Although rapamycin may have long

lasting effects on altering nociception, our data suggest that for this paradigm, post-

reactivation rapamycin blocked the reconsolidation of a conditioned fear memory in nonstressed controls without altering fear memory in chronically stressed rats.

In the current study, we investigated the functional activation of key limbic structures, especially the amygdala, following fear memory reactivation and PR-LTM in response to chronic stress and rapamycin treatment. We first demonstrated that *zif268* mRNA induction in the LA is specific to associative memory retrieval. The LA is known to be the initial point of convergence for CS and US input for fear memory formation and for the CS during retrieval (LeDoux, 2000; Johansen *et al.*, 2011). Both chronically stressed and nonstressed controls that were fear conditioned (CSUS) showed significant *zif268* mRNA induction in the LA compared to respective naïve control groups that were not conditioned (naïve). Furthermore, these fear memory reactivation data are relevant to recent findings that *zif268* is required for fear memory reconsolidation (Bozon *et al.*, 2003; Lee *et al.*, 2004; Maddox *et al.*, 2011), as reactivation determines the onset of reconsolidation processes. When looking closer into chronic stress and conditioning effects on relative levels of *zif268* mRNA in the amygdala, we observed an enhancement of *zif268* mRNA induction in both the LA and BA compared to nonstressed controls. These effects tend to correspond with our behavioral results, as chronic stress facilitated acquisition and enhanced freezing to tone during fear memory reactivation. In both cases, chronic stress enhanced *zif268* mRNA expression within the LA and BA, two critical amygdala subregions involved in associative fear. While the direction of chronic stress effects in the CSUS groups compared to the control CSUS group were similar in the BA to those seen in the LA, both nonstressed CSUS and naïve groups showed similar levels of *zif268* mRNA in the BA during fear memory reactivation, with significantly lower

levels in the stressed naïve group. Disparate BA effects in the naïve groups was unexpected, but this might suggest that fear conditioning alters *zif268* mRNA induction during fear memory reactivation specifically following chronic stress, and is consistent with previous findings on retrieval-induced Egr-1 (Zif268) and another plasticity-related IEG, Arc/Arg3.1, where expression was primarily restricted to the LA under nonstressed conditions (Maddox *et al.*, 2011; Maddox & Schafe, 2011). The BA is thought to be an integration site for emotional processing, and is not required for associative fear learning (Nader *et al.*, 2001), or perhaps reconsolidation. The observed chronic stress effects in this subregion however, are consistent with the structural enhancements observed in the entire basolateral (BLA) complex (Vyas *et al.*, 2002). Given the role of *zif268* and the LA in fear memory formation and that chronic stress sensitizes the stress response to subsequent novel stressors (Bhatnagar & Dallman, 1998), these data support the hypothesis that traumatic memories in PTSD may become "overconsolidated" under the influence of stress hormones stimulated by the traumatic experience (Pitman, 1989), particularly in chronically stressed populations.

We also showed significant enhancement of *zif268* mRNA expression within the LA and BA during post-retrieval long term memory. These findings are consistent with our *zif268* mRNA data for fear memory retrieval, and importantly with the behavioral outcomes showing chronic stress enhanced fear memories that are resistant to reconsolidation, with persistent enhancement of fear expression when tested for reconsolidation effects for long term memory. In contrast to our findings for *zif268* mRNA expression during fear memory retrieval, in the cohort tested for functional activation during PR-LTM we also showed a reduction in functional activation in dorsal

hippocampal CA1. The novel finding that chronic stress reduces hippocampal functional activation during fear memory retrieval is consistent with the regional structural changes we observe in the hippocampus following chronic stress (Sousa *et al.*, 2000; Hoffman *et al.*, 2011), and may reflect impaired general hippocampal function at this point in memory testing. However, we did not observe the same pattern of reduced CA1 activation when tested for initial fear memory activation (Table 1), which is consistent with a previous study showing enhanced CA1 *zif268* mRNA during retrieval of a contextual, but not cued fear memory (Hall *et al.*, 2001). Compared to those tested for memory reactivation (1 CS trial) the day after initial conditioning, groups that were tested in PR-LTM for *zif268* mRNA expression had undergone 6 CS additional CS trials (between PR-STM and PR-LTM testing), which may impact the influence of hippocampal involvement in fear memory representation. The chronic stress effect within hippocampal CA1 for fear memory retrieval-induced *zif268* mRNA is in contrast to a previous finding from our lab for fear extinction memory-induced Fos protein expression, a different IEG, where we showed chronic stress enhanced CA1 Fos expression (Hoffman *et al.*, under review). While in both studies we examined fear memory induced IEG expression similarly as a tool to determine differential patterns of functional activation, important differences between experiments and IEGs likely underlie the disparate outcomes. The previous study examined chronic stress effects on contextual fear extinction, a different learning paradigm, with little pre-exposure to the contexts unlike the current study, as contextual processing being a critical feature of hippocampal function (Fanselow & Dong, 2010). Furthermore, although these regulatory transcription factors (RTFs) are both induced by functional activation within neurons, fundamental

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differences between the two RTFs should be considered such as the constitutive expression of *zif268* and its potential bi-directional effects (Worley *et al.*, 1991) vs. the nearly absent basal levels of *c-fos* (Sagar *et al.*, 1988). Regardless of the direction of effect, it is apparent that chronic stress significantly impacts the amygdala-hippocampal network (Ghosh *et al.*, 2013), and alters functional activity during retrieval of fear memories (current study and Hoffman *et al.*, under review), similar to functional imaging differences in the human PTSD brain (Rauch *et al.*, 2006; Shin *et al.*, 2006).

To our knowledge, our study is the first to explore whether chronic stress enhanced fear memories undergo reconsolidation and can be vulnerable to disruption in a PTSD-like phenotype, with the ultimate goal of attenuating strong fear memories. However, we observed that these enhanced fear memories were unaffected by post-retrieval rapamycin, unlike in the nonstressed control conditions. Many studies that aim to block reconsolidation of fear memories target the restabilization phase, which requires protein synthesis. Intra-amygdala infusion or systemic administration of amnestic agents such as agents that inhibit protein synthesis, protein translation (such as rapamycin in the current study), RNA synthesis, kinase signaling activity, and others (see Nader & Einarsson, 2010 for review) have shown to be effective at producing persistent amnesia for the original memory. However, like our data and in other studies (Suzuki *et al.*, 2004; Glover *et al.*, 2010; Muravieva & Alberini, 2010), not all memories seem to undergo reconsolidation, and therefore produce null results in attempts for disruption. These constraints on reconsolidation are known as boundary conditions. Strong training protocols have demonstrated memories resistant to reconsolidation (Suzuki *et al.*, 2004; Wang *et al.*, 2009) and here we identify chronic stress enhanced fear as a new boundary

condition in fear memory reconsolidation under the tested parameters. While protein synthesis is required for reactivated memories to restabilize, synaptic protein degradation is a critical upstream event required for memories to be destabilized once retrieved (Lee *et al.*, 2008). It has been hypothesized that retrieved memories that do not undergo reconsolidation are not destabilized, which is necessary for reactivated memory lability (Finnie & Nader, 2012), and therefore resistant to amnestic agents in subsequently impairing the original memory. Several mechanisms have been identified to play a role in the regulation of memory destabilization (see Finnie & Nader, 2012 for review). For instance, strong training protocols downregulate BLA NR2B NMDA-receptor subunit expression that demonstrates to be resistant to reconsolidation (Wang *et al.*, 2009). Another study showed that activation of NMDA receptors with $_D$-cycloserine prior to memory reactivation prepares the neural substrates for memory destabilization to occur (Bustos *et al.*, 2010) and enables pharmacological reconsolidation blockade. Identifying the putative molecular mechanisms of memory destabilization and boundary conditions in reconsolidation can allow us to better access the original memory and facilitate reconsolidation blockade for its therapeutic application in a PTSD-like phenotype, such as in chronic stress enhanced fear memories.

A core feature of PTSD is that the memory of a traumatic event is persistent, unwavering and characterized by excessive strength (Pitman, 2011). Furthermore, only a subset of trauma victims ultimately develops the disorder (Breslau *et al.*, 1991), indicating individual differences in the susceptibility to develop PTSD following exposure to a traumatic event, with prior chronic stress as a potential environmental risk factor. Our model involving chronic stress and fear conditioning supports a PTSD-like

phenotype in that we demonstrate persistent and strong fear memories as evidenced in the current work (reconsolidation-resistant) and previous studies (extinction-resistant; Baran *et al.*, 2009; Hoffman *et al.*, 2010; Hoffman *et al.*, under review). We further demonstrate face validity with chronic stress and fear conditioning as an appropriate model for PTSD, with parallel outcomes to what is observed in humans (Rauch *et al.*, 2006; Shin *et al.*, 2006) with functional changes in key limbic structures including enhanced amygdala and decreased hippocampal IEG induction during fear memory recall. The observed chronic stress enhanced fear memories that are reconsolidation-resistant pose a challenge for strategies that target reconsolidation blockade to treat clinical symptoms of traumatic fear memories in PTSD. Nonetheless, the current study provides an initial step in the direction to explore this promising novel mechanism to indelibly weaken traumatic memories in a PTSD-like phenotype. Collectively, our data support the hypothesis that chronic stress creates a vulnerability to develop maladaptive fear after a traumatic event though functional alterations in fear neurocircuitry, and offers an appropriate model to study neurobiological mechanisms associated with the susceptibility to and consequences of PTSD fear memory in hopes to find more effective treatments.

CHAPTER 5

GENERAL DISCUSSION

This dissertation summarized a series of experiments designed to understand basic preclinical mechanisms of limbic brain plasticity following chronic stress in the context of vulnerability for PTSD. A triad of interconnected limbic structures was targeted, the hippocampus, mPFC and amygdala, which together might contribute to the pathogenesis of exaggerated fear following chronic stress. In particular, chronic stress appears to create a dysregulated network among these three brain structures, as demonstrated by different behavioral investigations using the RAWM for hippocampal function and fear conditioning for amygdala and mPFC mediated-behaviors. A variety of molecular techniques to investigate how chronic stress impacts limbic plasticity were utilized that included Golgi stain analysis for structural dendritic morphology, immunohistochemistry and *in situ* hybridization for IEG induction to measure functional activation. Golgi analysis and IEG measures allowed us to capture a "snapshot" for both structural and functional plasticity within localized brain tissue. Chapter 2 demonstrated that immediately following chronic restraint stress, rats exhibited poor spatial working and reference memory with corresponding hippocampal CA3 dendritic retraction. However, when allowed sufficient time to recover (21d following the end of stress), previously stressed rats showed improved spatial memory that corresponded to restored hippocampal CA3 dendritic complexity to that of control levels. Chapter 3 described a study that investigated whether chronic stress affects nonassociative fear during auditory fear extinction. Chronic stress impaired mPFC-mediated extinction memory, and impacted nonassociative fear by enhanced contextual fear during extinction that generalized to a

new context. Chronic stress also enhanced functional activation in the hippocampus and amygdala during fear extinction memory retrieval. Finally in chapter 4, a series of studies were conducted to determine whether chronic stress enhanced fear memories were vulnerable to disruption by manipulating memory reconsolidation. Targeting reconsolidation processes was a novel approach to weaken stress-induced strong fear memories. Chronic stress enhanced fear conditioning and elevated amygdala functional activation during fear memory reactivation, and these enhanced fear memories were resistant to reconsolidation. Collectively, these studies demonstrated the plastic and dynamic effects of chronic stress on limbic neurocircuitry implicated in PTSD. We showed that chronic stress created a structural and functional imbalance across the hippocampus, mPFC, and amygdala, which lead to a PTSD-like phenotype with weakened hippocampal and mPFC function and hyperactive amygdala function that might have contributed to persistent and exaggerated fear following fear conditioning.

The studies presented in this dissertation provided support that the hippocampus is vulnerable to changes induced by chronic stress, and these changes may in part underlie a predisposed vulnerability for a PTSD-like phenotype following a traumatic event. Decreased hippocampal volume is a risk factor for the susceptibility for PTSD (Gilbertson *et al.*, 2002). The data presented in chapter 2 provided objective evidence of how chronic stress can structurally impact the hippocampus by CA3 dendritic retraction that corresponded to functional deficits in hippocampal-mediated spatial memory. However, these chronic stress induced hippocampal functional deficits were only observed within the window of CA3 dendritic retraction, while a recovery period restored CA3 dendritic complexity and potentially benefited hippocampal function, suggesting

potential resilience factors. Given the dynamic plasticity of the hippocampus and response to chronic stress and recovery and that reduced hippocampal volume is a risk factor for PTSD, there may be a window of opportunity after chronic stress for a traumatic experience to result in exaggerated and persistent fear. Although not tested in our study, it is possible that learned fear within the dendritic retraction window is highly susceptible for PTSD-like behavior, but less susceptible 21d later and warrants future investigation. Furthermore, it is well documented that fear extinction is context dependent and requires an intact hippocampus (Corcoran & Maren, 2001; Corcoran *et al.*, 2005). In chapter 3 we showed enhanced contextual fear during extinction and generalization to a novel context in our chronically stressed groups, which supports a PTSD-like phenotype that likely reflects compromised hippocampal function. In chapters 3 and 4 we also showed differential hippocampal CA1 IEG induction during retrieval of a fear memory. These studies collectively suggest that chronic stress induced hippocampal dysregulation contributes to altered fear memory and extinction processing.

The mPFC is another limbic structure implicated in PTSD that may be more sensitive than the hippocampus in terms of rapid plastic changes by mild and acute stressors. For instance, the mPFC is the first structure within this circuitry to show experience-dependent differential IEG induction one day after exposure to restraint stress (Hoffman *et al.*, 2013). Also, the mPFC demonstrates dendritic retraction to mild stress including repeated injections of saline after only one week (Brown *et al.*, 2005), which contrasts to the several weeks required to demonstrate dendritic retraction in the CA3 region of the hippocampus (McLaughlin *et al.*, 2007). In chapters 2 and 3, the functional ramifications related to chronic stress effects on mPFC-mediated behaviors were

delineated and these included impaired working memory (chapter 2) and poor fear extinction memory (chapter 3). The latter finding is relevant to the PTSD-like phenotype for exaggerated and persistent fear memory. In line with our behavioral observations, PTSD patients also show deficits in extinction retention (Orr *et al.*, 2000). The mPFC is well established to be required for the storage and retrieval of fear extinction (Maren & Quirk, 2004; Quirk & Mueller, 2008), and it is evident that PTSD patients show reduced activity within the mPFC (Liberzon & Martis, 2006). Relevant to the challenges in treating persistent fear in patient populations (Pitman *et al.*, 1996), we and others have demonstrated robust spontaneous recovery following fear extinction in chronically stressed rats (Miracle *et al.*, 2006; Baran *et al.*, 2009; Wilber *et al.*, 2011; Hoffman *et al.*, under review), that has recently been shown to correspond with alterations in disruptions in CS-evoked neuronal activity in the chronically stressed mPFC (Wilber *et al.*, 2011). More research is needed to determine the neurobiological consequences of chronic stress on the mPFC in the context of fear extinction retrieval deficits.

The amygdala is exquisitely sensitive to stressors, drives the integration of emotional stimuli (LeDoux, 2000), and has shown to be highly implicated in PTSD. Chronic stress causes hyperexcitability (Rosenkranz *et al.*, 2010) and enhances dendritic arborization within the amygdala that corresponds with increases in anxiety-like behavior (Vyas *et al.*, 2002). Moreover, these chronic stress induced structural changes tend to persist beyond the time point that the hippocampus and mPFC have shown structural recovery (21d; Vyas *et al.*, 2004). Also, exposure to an acute aversive experience such as immobilization stress (Mitra *et al.*, 2005) or acute glucocorticoid administration (Mitra & Sapolsky, 2008) causes delayed increases in dendritic structure within amygdala neurons,

emphasizing amygdala sensitivity to stress. The sensitive and indelible acute and chronic stress induced structural reorganization within amygdala neurons may have implications for delayed onset PTSD and add to the complexity of the disorder with resilience resistant factors. The dynamic structural alterations within the hippocampus and amygdala following chronic stress and their implications in PTSD prompts the question as to whether the state of the hippocampus or amygdala underlies the susceptibility for PTSD at the time of a traumatic event. A recent study suggests that the lasting effects of chronic stress on amygdala excitability underlie enhanced fear responses 60 days following the end of chronic stress (Burgos-Robles & Goosens, 2013), well beyond the period for hippocampal recovery (Conrad *et al.*, 1999; Sousa *et al.*, 2000; Hoffman *et al.*, 2011). Investigations of the neural mechanisms of learned fear indicate that the amygdala is required for the acquisition, storage, and expression of conditioned fear (LeDoux, 2003). Exaggerated fear in response to a traumatic event is a core feature of PTSD, which may involve augmented amygdala activity (Yehuda & LeDoux, 2007). Our behavioral and IEG data demonstrate the functional consequences on amygdala driven fear memories, as chronic stress enhanced overall freezing behavior and amygdala functional activation in chapters 3 and 4. We also showed that chronic stress-enhanced fear memories were resistant to extinction and reconsolidation blockade in distinct efforts to reduce conditioned fear expression in a PTSD-like phenotype.

It is noteworthy that the hippocampus, mPFC, and amygdala are part of an interconnected network involved in cognitive and emotional processing. With different behavioral paradigms, the functional consequences following chronic stress can be explored for each structure. For example, spatial performance can be used to assess

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hippocampal function, working memory for mPFC function, and fear conditioning can be implemented to understand amygdala involvement. However, in chronically stressed animals as well as in PTSD patients, there are alterations within the entire integrated limbic network and in similar directions that involves a hyperresponsive amygdala and hyporesponsive hippocampus and mPFC. The fear extinction circuit is a good example of limbic system integration that can become disrupted after chronic stress and in PTSD. While the mPFC has shown to be required for the retention and recall of fear extinction (Quirk *et al.*, 2006), which is disrupted following chronic stress (Wilber *et al.*, 2011), it unlikely to be sole influence driving the behavioral impairment. In fact, extinction learning and expression relies on an intact network of the hippocampus, mPFC, and amygdala (illustrated in Fig. 1), all of which are impacted by chronic stress. Specifically, each structure receives inputs from CS information, as the mPFC integrates CS information and contextual information from the hippocampus. Within the extinction context, the mPFC inhibits amygdala output, thereby lessening fear expression. When structures in this network become disrupted following chronic stress, we observe behaviors that reflect exaggerated fear, extinction retention deficits, and disrupted contextual integration with fear generalization in novel contexts, as observed in chapter 3 of this dissertation. These behavioral disruptions reflect an imbalance between hippocampal and mPFC regulation in favor of amygdala function overdrive, and is in line with chronic stress induced structural imbalance as well as in PTSD symptomology and functional brain imaging. Fig. 21 illustrates chronic stress induced dysregulated limbic network in the context of the collectively observed morphological, functional, and behavioral disruptions.

Taken together, the current studies presented and discussed in this dissertation add to the growing literature on chronic stress induced plastic changes within key limbic brain areas that are implicated in PTSD. We supported the hypothesis that chronic stress induced structural and functional alterations create a vulnerability to develop maladaptive fear following a traumatic event by representing reduced hippocampal and mPFC function in conjunction with enhanced amygdala activation and exaggerated fear following chronic stress and fear conditioning.

The general paradigm of chronic stress prior to fear conditioning provides a novel way of using a familiar paradigm for the investigation of the underlying neurobiological mechanisms of exaggerated fear memories present in PTSD that includes hippocampal and mPFC hypofunction and amygdala overactivation. Decades of research have provided a fundamental basis for understanding the mechanism that underlies formation and maintenance of fear memories (LeDoux, 2000; 2003; Johansen *et al.*, 2011), which lies at the core of traumatic memory mechanisms in PTSD. Applying Pavlovian fear conditioning paradigms in conjunction with what we know about how these limbic circuits processes fear memories provides a powerful tool in our pursuit for understanding how these circuits become dysregulated in PTSD. Since PTSD does not develop in all those who experience a traumatic stressor, this suggests underlying individual differences in predisposed resilience and vulnerability factors. Moreover, most preclinical research investigating traumatic memory mechanisms and novel therapeutic approaches involve animals with an undisrupted system and intact adaptive processes (i.e., nonstressed controls), where those relevant to PTSD are not typical, but exaggerated responses. Our model hones in on this niche, as chapters 3 and 4 provided evidence for

chronic stress and fear conditioning in rats to produce a PTSD-like phenotype with behavioral data that parallel the symptomology and challenges faced in treating pathological fear and functional imaging data that correspond to clinically observed abnormalities within the limbic circuit, demonstrating strong face validity. This paradigm also provides convincing construct validity by taking advantage of well established and widely used measures such as chronic restraint stress (Pare & Glavin, 1986; McEwen, 2000), Pavlovian fear conditioning and defensive responding in rats as an index of fear (Pavlov, 1927; Fanselow, 1994), and functional activation mapping with IEG expression quantification in response to synaptic plasticity (Sagar *et al.*, 1988; Cole *et al.*, 1989; Morgan & Curran, 1991). More research utilizing this paradigm is needed to establish predictive validity with current accepted pharmacological therapies. Although as a caveat, our behavioral phenotype represents treatment-resistant exaggerated and persistent fear as demonstrated by extinction learning and memory deficits (chapter 3) and resistance to reconsolidation (chapter 4). It would be interesting to combine currently accepted pharmacological treatments in conjunction with extinction in our PTSD-like phenotype, such as the NMDA partial agonist, D-cycloserine as it shows promise in clinical trials to facilitate extinction learning when utilized in the context of exposure therapy (Norberg *et al.*, 2008). Therefore, our paradigm provides a vulnerability factor that is essentially unexplored in other studies of fear memories in relation to PTSD, may be useful tool to understand the etiology of clinical condition and warrants further application in understanding the neurobiology of and potential therapeutic targets for PTSD.

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Table 1. Chronic stress and fear conditioning effects on fear memory reactivation zif268

mRNA expression within amygdala and hippocampal subregions.

ROI	CON-CSUS	CON-naïve	STR-CSUS	STR- naïve
LA	**0.271 ± 0.019*****	0.196 ± 0.013	**0.332 ± 0.015^ *****	0.193 ± 0.020
BA	0.176 ± 0.014	**0.178 ± 0.011 #**	**0.228 ± 0.017 ^**	0.132 ± 0.012
CeL/C	0.159 ± 0.011	0.145 ± 0.012	0.175 ± 0.009	0.151 ± 0.009
CeM	0.137 ± 0.024	0.139 ± 0.012	0.178 ± 0.012	0.154 ± 0.016
MeA	0.122 ± 0.013	0.134 ± 0.008	0.150 ± 0.012	0.130 ± 0.009
CA1	0.416 ± 0.023	0.447 ± 0.030	0.405 ± 0.022	0.445 ± 0.022
CA3	0.220 ± 0.015	0.221 ± 0.016	0.223 ± 0.012	0.218 ± 0.012
DG	0.216 ± 0.015	0.201 ± 0.018	0.213 ± 0.013	0.214 ± 0.008

Results of two-way ANOVAs for stress and conditioning and t-tests for stress effects for conditioned groups on µCi/g (± SEM). ***$p<0.001$ main effect of conditioning for CSUS vs. naïve; ^$p<0.05$ significant effect for STR-CSUS vs. CON-CSUS; #$p<0.05$ for CON-naïve vs. STR-naïve. BA: basal amygdala, CA1: *Cornu Ammonis* 1, CA3: *Cornu Ammonis* 3, CeL/C: central lateral/central amygdala, CeM: central medial amygdala, CON: nonstressed control, CSUS: conditioned, DG: dentate gyrus, LA: lateral amygdala, MeA: medial amygdala, STR: chronic stress.

Table 2. Chronic stress and post reactivation rapamycin treatment post-reactivation long term memory zif268 *mRNA expression within amygdala and hippocampal subregions. Data are represented as mean gray value ± SEM*

ROI	CON-VEH	CON-RAPA	STR-VEH	STR- RAPA
LA	0.188± 0.024	0.159 ± 0.008	**0.252 ± 0.024 ^^**	**0.245 ± 0.030 ^^**
BLA	0.135 ± 0.017	0.113 ± 0.014	**0.163 ± 0.010 ^**	**0.151 ± 0.019 ^**
CeL/C	0.074± 0.016	0.071 ± 0.014	0.083 ± 0.011	0.078 ± 0.013
CeM	0.067 ± 0.015	0.059 ± 0.019	0.053 ± 0.009	0.057 ± 0.011
MeA	0.056 ± 0.016	0.045 ± 0.015	0.058 ± 0.009	0.046 ± 0.013
CA1	**0.401 ± 0.019 ^**	**0.409 ± 0.017 ^**	0.348 ± 0.027	0.356 ± 0.015
CA3	0.184 ± 0.012	0.169 ± 0.022	0.163 ± 0.013	0.149 ± 0.014
DG	0.128 ± 0.007	0.106 ± 0.014	0.110 ± 0.013	0.115 ± 0.011

Results of two-way ANOVAs for stress and drug condition on µCi/g (± SEM). ^^p<0.01; ^p<0.05 main effect for STR vs. CON. BA: basal amygdala, CA1: *Cornu Ammonis* 1, CA3: *Cornu Ammonis* 3, CeL/C: central lateral/central amygdala, CeM: central medial amygdala, CON: nonstressed control, DG: dentate gyrus, LA: lateral amygdala, MeA: medial amygdala, RAPA: rapamycin, STR: chronic stress, VEH: vehicle.

Table 3. Effects of chronic restraint on body weight gain. Data are represented as weight in grams (g) ± S.E.M. CON=control, STR = stress.

Cohort/ Stress Condition (n)		Baseline	Day 7	Day 14	Day 21
1	CON (16)	308.6 ± 1.6	365.4 ± 2.7	403.3 ± 3.3	429.9 ± 4.1
	STR (16)	309.1 ± 2.5	335.4 ± 3.4	364.6 ± 4.1	379.8 ± 4.0
2	CON (16)	323.2 ± 3.5	375.4 ± 3.3	416.3 ± 4.6	445.3 ± 5.6
	STR (16)	322.1 ± 3.5	341.8 ± 2.8	365.4 ± 3.2	388.4 ± 3.5
3	CON (20)	311.1 ± 4.0	363.2 ± 4.5	395.6 ± 5.1	420.7 ± 6.2
	STR (20)	317.8 ± 5.1	323.8 ± 4.0	360.2 ± 5.3	366.1 ± 5.3
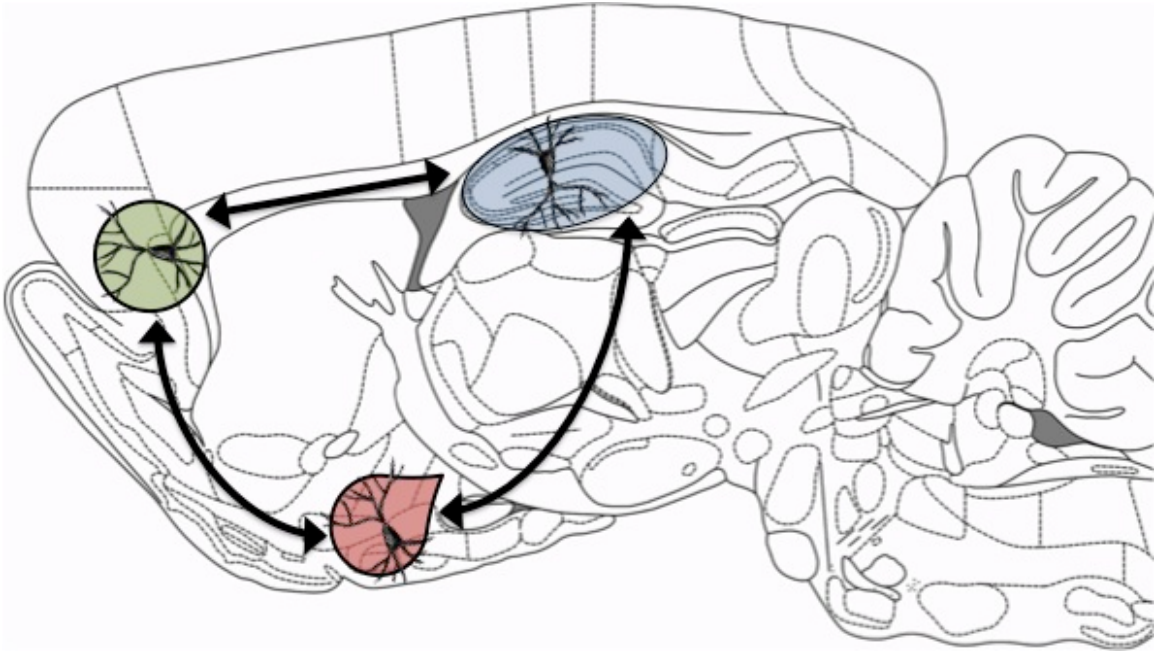
Figure 1. Reciprocal hippocampal-mPFC-amygdala limbic network. Schematic sagittal view of a rat brain adapted from Paxinos and Watson (2007) depicting the structural and functional network under healthy conditions. The hippocampus, mPFC, and amygdala are interconnected by excitatory projections that integrate and process emotional stimuli that contribute to the formation and regulation of emotional memories.

CON	No restraint 42d	
STR-IMM	No Restraint 21d	Restraint 6h/d/21d
STR-DEL	Restraint 6h/d/21d	No Restraint 21d

2 day RAWM Testing

Trials: 1-12 ⟷1h⟩ 13-18 ⟷24h⟩ RT

Blocks: 1-6 7-9
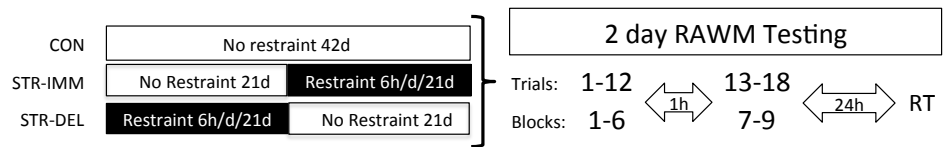
Figure 2. Experimental timeline. Animals were divided into three groups: nonstressed control left undisturbed throughout the study (CON), chronically stressed restrained 6h/d/21d for the second half of the study tested and sacrificed within two days after the end of restraint (STR-IMM), and chronically stressed restrained for 6h/d/21d during the first half of the study tested and sacrificed with a 23d post stress delay (STR-DEL). Two-day radial arm water maze (RAWM) testing where day 1 consisted of 12 massed trials analyzed in blocks of 2 (acquisition), a one hour inter-trial-interval, and an additional 6 trials (3 blocks, relearning). Twenty-four hours later, all animals were given a retention trial (RT) and were sacrificed within an hour of testing and processed for Golgi-staining.

A. RAWM: Reference Memory

B. RAWM: Working Memory

C. 24h Retention Trial: Reference Memory

D. 24h Retention Trial: Working Memory
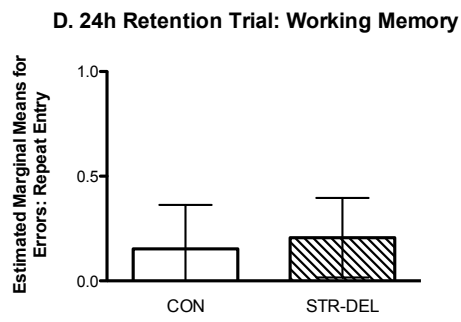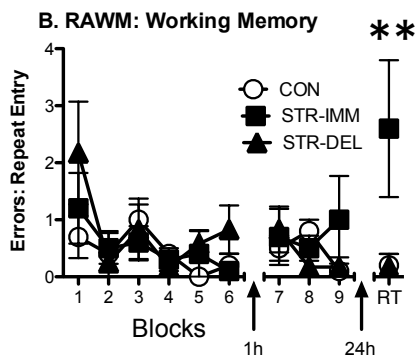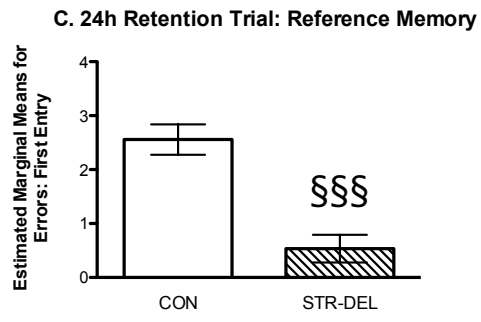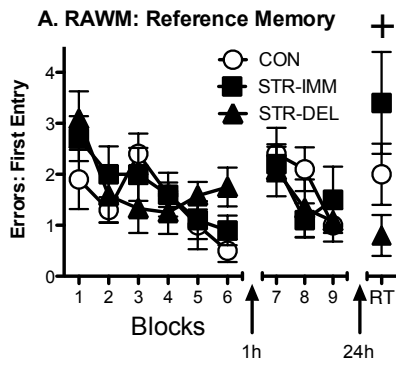
Figure 3. Recovery of chronic stress-induced spatial memory deficits in a radial arm water maze task (RAWM). (A) For first time entry errors, all groups acquired the RAWM task over the first 12 trials, and second 6 trials after a 1h delay. After a 24h delay, STR-IMM made more first time entry errors than did STR-SEL. (B) For repeat entry errors, all groups acquired the RAWM task over the first 12 trials, and second 6 trials after a 1h delay. After a 24h delay, STR-IMM made more repeat entry errors than did STR-DEL and CON. (C) Estimated marginal means for CON and STR-DEL from ANCOVA analysis for 24h retention trial (RT) with the mean of the first 12 trials (first 6 blocks) as a covariate. STR-DEL had fewer first time entry errors during RT compared to CON. (D) Estimated marginal means for CON and STR-DEL from ANCOVA analysis for 24h retention trial (RT) with the mean of the first 12 trials (first 6 blocks) as a covariate. STR-DEL and CON had similar repeat entry errors during RT. Data are represented as means $\pm$ S.E.M. * p=.05 for STR-IMM vs. STR-DEL, ** p<.01 STR-IMM vs. STR-DEL and CON., *** p<.001 compared to CON.

A. Apical Branch Points
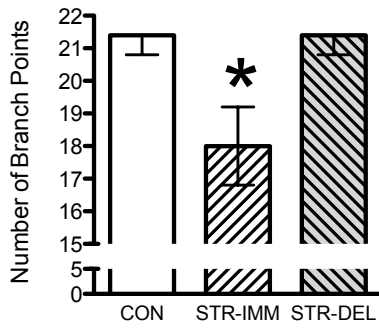
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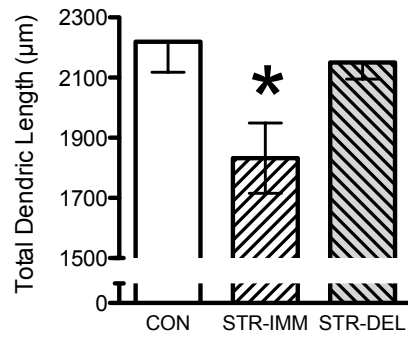
C. Basal Branch Points
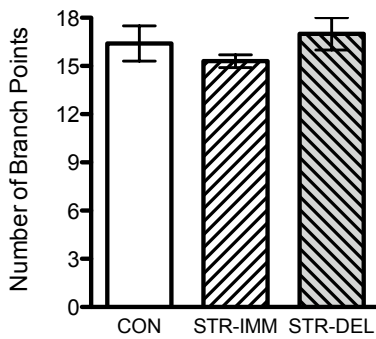
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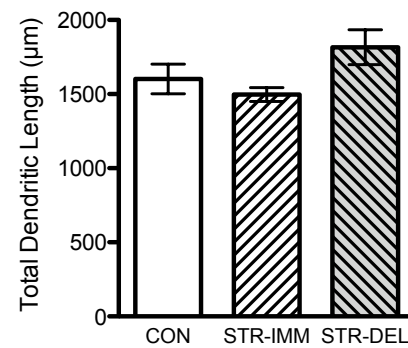
E. CON
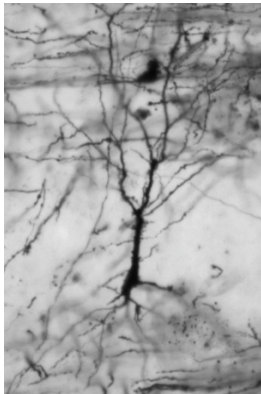
F. STR-IMM
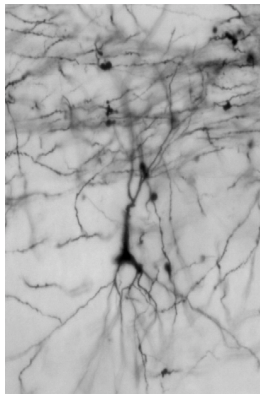
G. STR-DEL
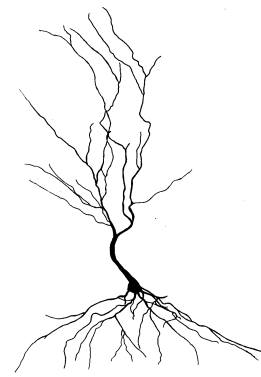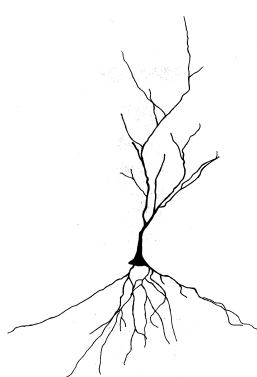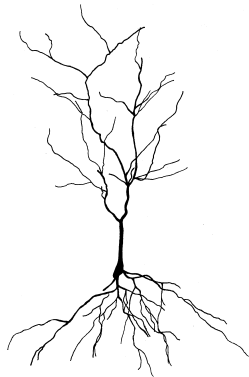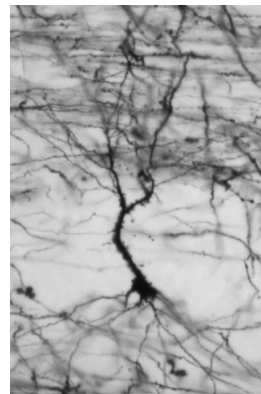
Figure 4. Recovery of hippocampal CA3 apical dendritic retraction. (A) Chronic stress without a delay (STR-IMM) showed significantly fewer apical dendritic branch points compared to nonstressed controls (CON) and chronic stress given a 21d post-stress delay (STR-DEL). (B) STR-IMM also showed a reduction in overall apical branch length compared to CON and STR-DEL. (C-D) There were no significant effects for basal - dendritic properties. (E-G top panel) Photomicrographs (20x magnification) showing representative Golgi-stained pyramidal neurons of the CA3 region of the hippocampus from each group. (bottom panel) Camera Lucida drawings taken from respective Golgi-stained CA3 cells. These images (E-G) represent dendritic properties of each experimental group. Note the decrease in apical dendritic arbors in the STR-IMM group compared to both CON and STR-DEL groups. Also note the restoration of apical dendritic complexity in the STR-DEL group to levels of CON. Data are represented as mean ± S.E.M. *p<.05 compared to CON and STR-DEL.
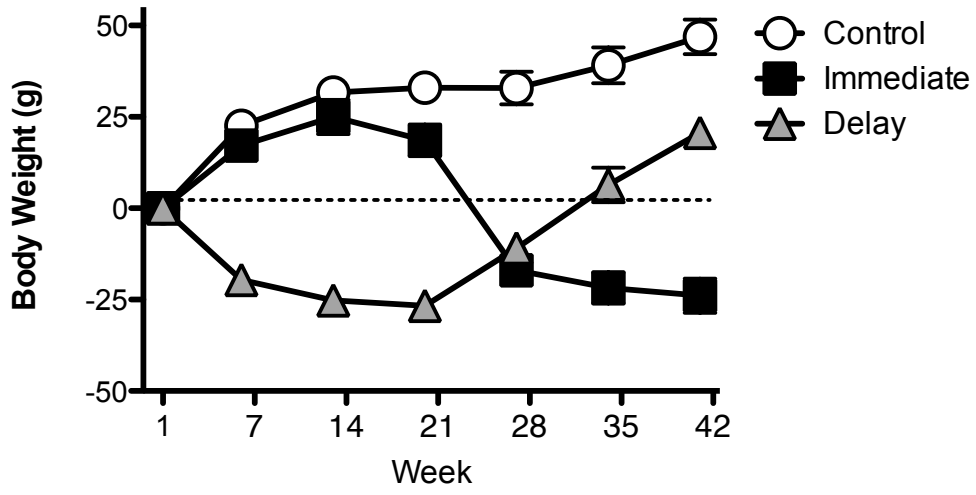
Figure 5. Change in body weight throughout the study. Chronic stress was an effective stressor as evidenced by attenuated body weight gain during the duration of restraint for each of the stressed groups. STR-IMM showed initial body weight gain and a reduction in body weight gain during the 21d of restraint. Similarly, STR-DEL showed initial reduction in body weight gain throughout restraint and gained weight after restraint ended. CON gained weight throughout the study. §§§ p<.001 for STR-DEL compared to STR-IMM and Control during the first three weeks, ‡‡‡ p < .001 for statistical difference for all three groups during the last three weeks (days 21 to 42). Data are represented as mean ± S.E.M. Note: in cases whereby error bars are not visible indicates that the error bars are smaller than the data point for the mean.

A.

Open Field → 24h → **Chronic Restraint 6h/d/21d** → 24h → Chamber Acclimation (Context A) → 24h → Conditioning (Context A) → 24h → Extinction 1 (Context A) → 24h → Extinction 2 (Context A) → 24h → SAME (Context A) / NOVEL (Context B) → c-Fos IHC
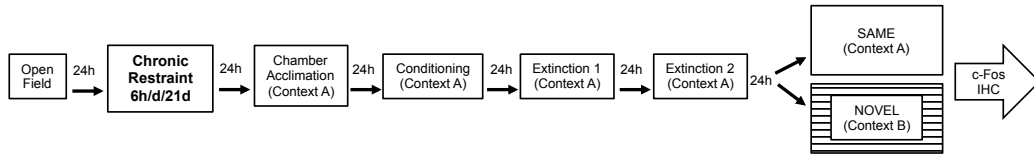
B.
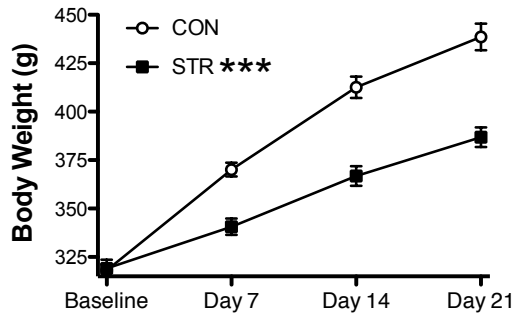
C.
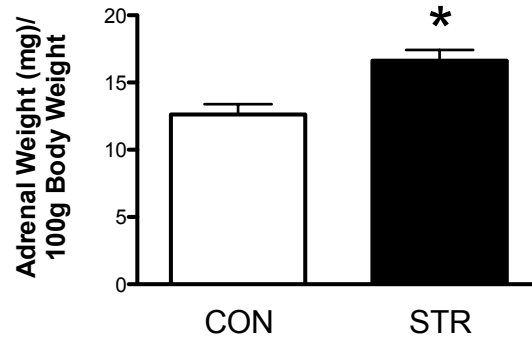
Figure 6. Timeline and Stressor Effectiveness. (A) Experimental Timeline. All groups were tested on the open field then the following day were either subjected to chronic wire mesh restraint (6h/d/21d) or handled briefly. Following chronic restraint, rats were acclimated to the conditioning chamber (Context A), and the next day were trained with 3x tone CS-footshock pairings. The next two days (Extinction 1 and 2, respectively), both groups underwent cued fear extinction with 15 CS-alone trials in Context A. Then the next day, both groups were either tested (6x CS) in the same (Context A) or a novel context chamber (Context B) and were euthanized 90min later and brains were processed for Fos immunohistochemistry. (B). Chronic restraint stress attenuated body weight gain across 21d of wire mesh restraint. (C) Chronically stressed rats had significantly greater adrenal weight per 100g of body weight. Data are represented as mean ± SEM; n=9-10/group. ***$p<0.001$ vs. CON on Days 7, 14, and 21. *$p<0.05$ vs. CON.

A.

% Freezing to CS

- CON
- STR

Conditioning Trial 3

% Freezing to CS

CON   STR

Conditioning Trials

B.

% Freezing to CS

C.

% Freezing to Context

Extinction 1
(3 trial blocks)

Extinction 2
(3 trial blocks)
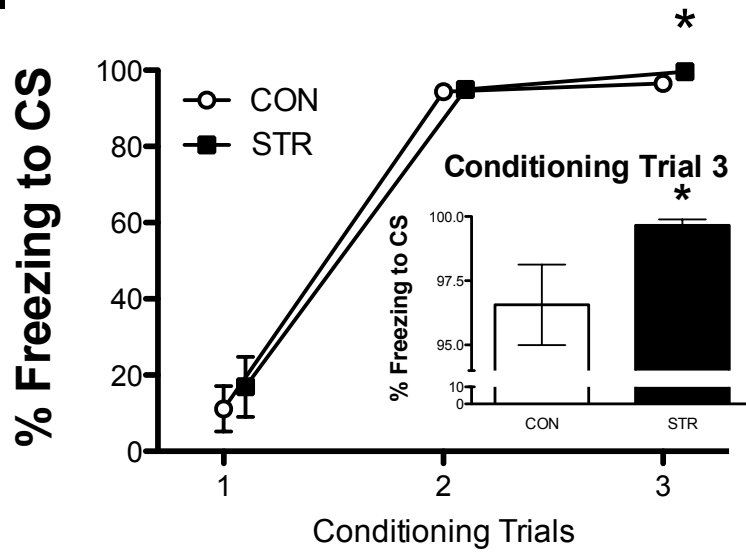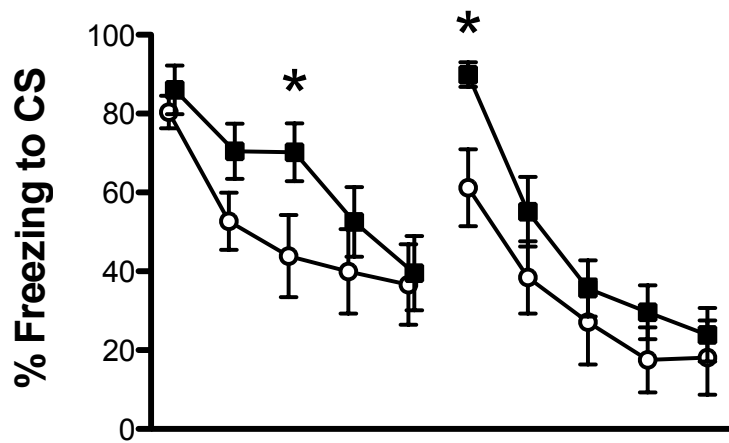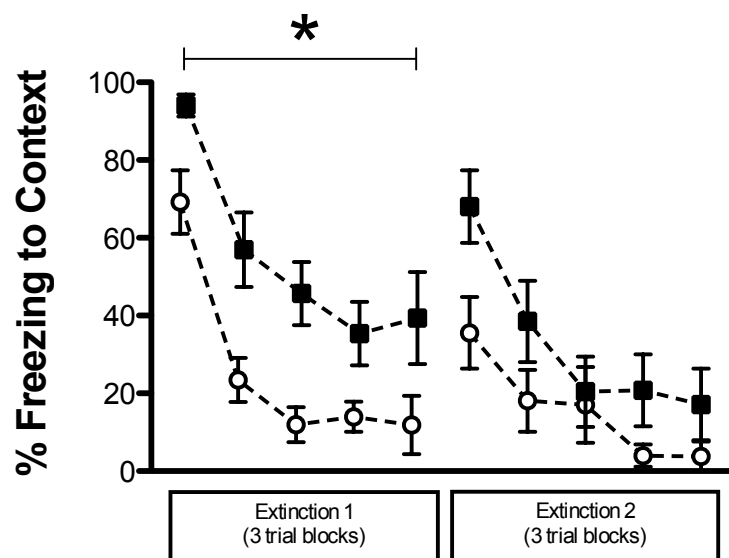
Figure 7. Conditioning and CS and Context Extinction. (A) Freezing to CS across conditioning trials, all groups showed greater freezing across conditioning trials, whereas chronic stress enhanced fear acquisition as evidenced by increased freezing to CS during trial 3 of conditioning (inset). (B) CS Fear Extinction. Chronic stress (STR) slowed extinction acquisition, demonstrated by increased freezing to CS midway through Extinction 1. STR also showed impaired extinction retention, with robust freezing at the beginning of Extinction 2 compared to CON. (C) Contextual Fear Extinction. STR had significantly greater contextual fear during Extinction 1 compared to CON. Data are represented as mean ± SEM; n=9-10/group. *p<0.05 vs. CON.
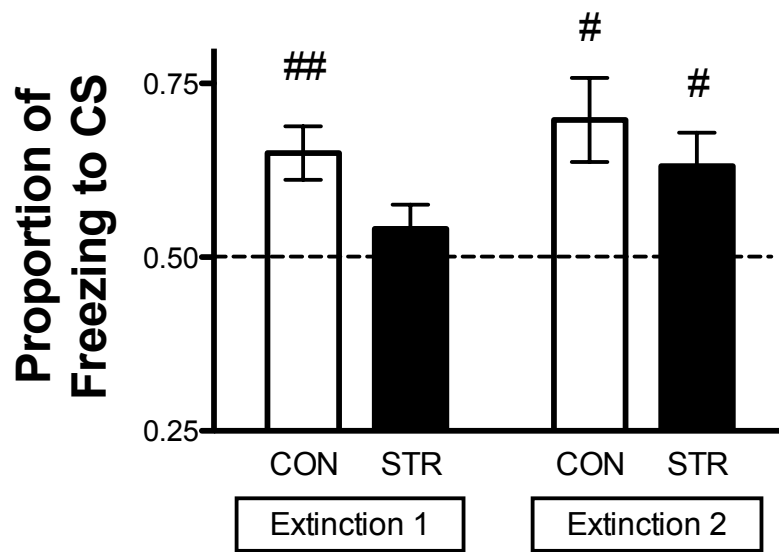
Figure 8. Proportion of Freezing to CS. During Extinction 1, CON displayed

significantly increased freezing in response to the CS than the background, as indicated

by a significantly higher proportion of freezing to CS compared to chance (0.5), while

STR displayed equivalent freezing to the CS and context. Both groups displayed

increased freezing to CS than background during Extinction 2. Data are represented as

mean ± SEM; n=9-10/group. ##$p<0.01$ vs. chance (0.5); #$p<0.05$ vs. chance (0.5).

(A)
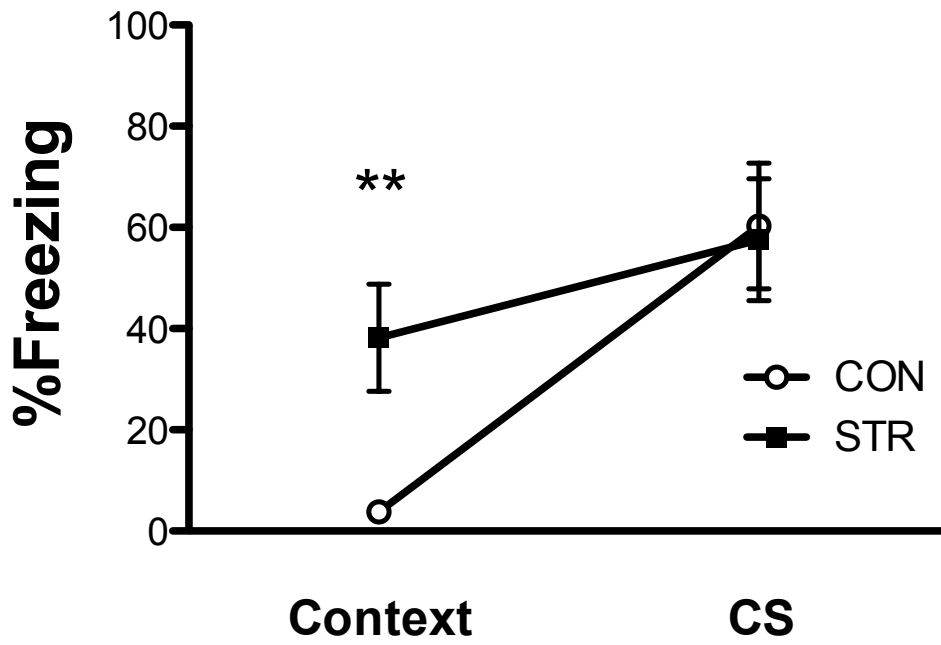
(B)
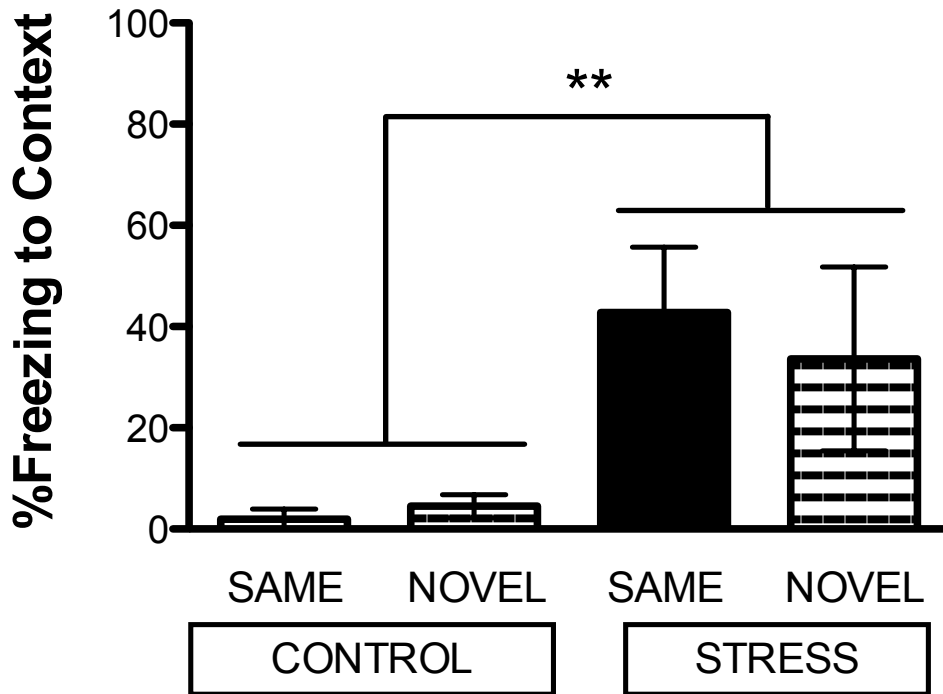
Figure 9. Chronic stress induces fear generalization to a novel context. (A) Chronic

stress enhanced freezing to context prior to the first CS presentation when tested for

extinction memory, while all groups showed similar levels of freezing during the first CS

presentation. There was no impact of test context (SAME and NOVEL collapsed within

each stress condition; n=8-10/group). (B) Fear Generalization. STR displayed increased

freezing to the context prior to trial 1, regardless of test context (SAME or NOVEL; n=4-

5/group). Data are represented as mean ± SEM; **p=0.01 vs. CON.

A.

B.

C.
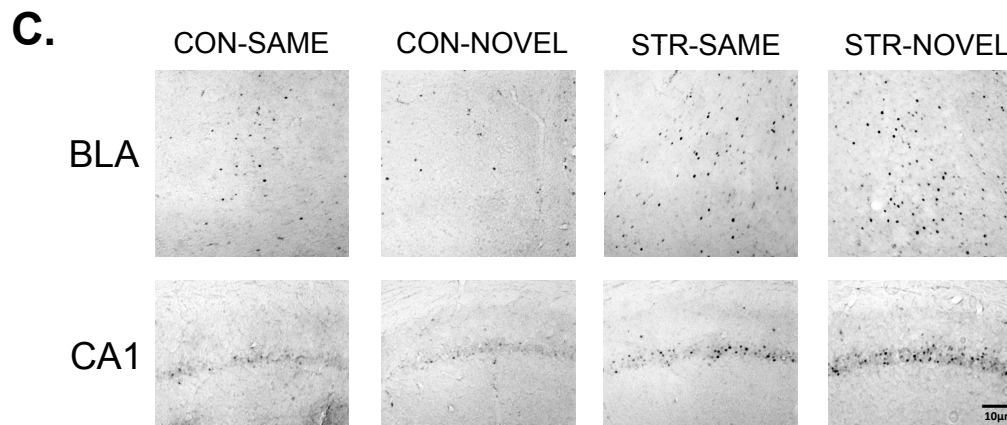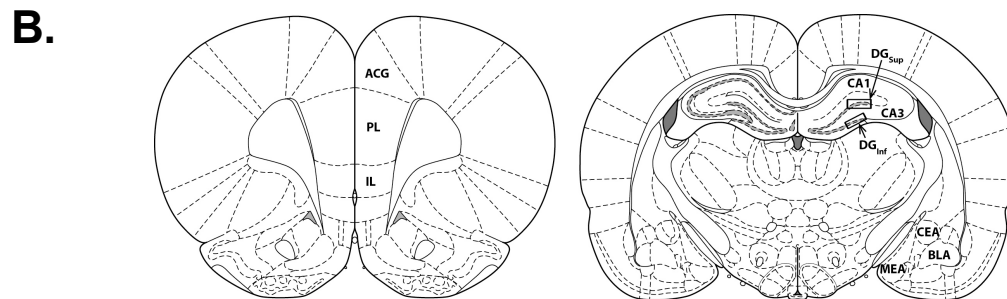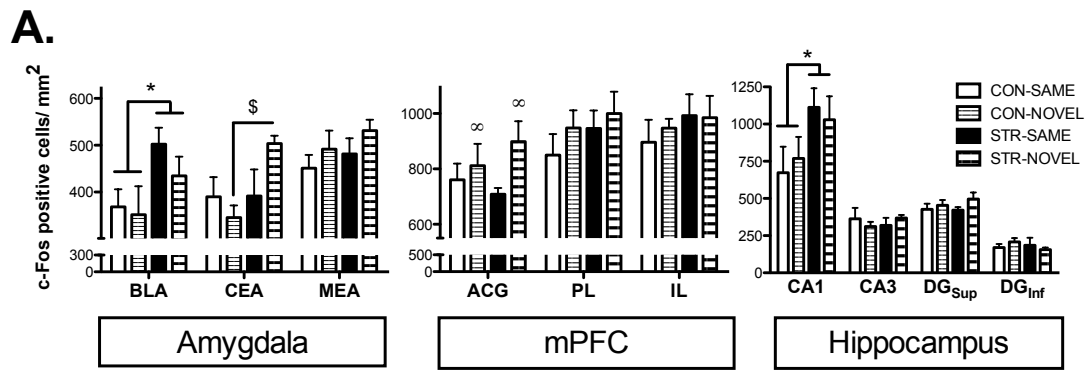
Figure 10. Fos IR labeling: Amygdala, mPFC, Hippocampus. (A) Regardless of test context, STR showed greater Fos IR compared to CON in amygdala BLA and dorsal hippocampus CA1. When tested in a novel context, STR showed increased Fos IR in amygdala CEA compared to CON. Regardless of stress history, when either group was tested in a novel context, Fos IR was in the mPFC ACG. (B) Regions of interest in coronal sections based on the rat brain atlas by Paxinos and Watson (2007). (C) Representative Fos photomicrographs observed in the BLA and CA1. Data are represented as mean ± SEM; n=4-5/group. *p<0.05 vs. CON; $p<0.001 vs. CON-NOVEL; ∞p<0.05 vs. SAME.
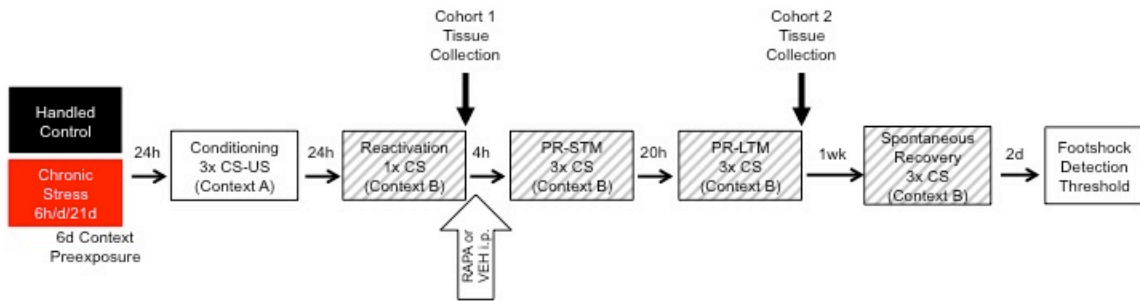
Figure 11. Experimental timeline. Rats were either chronically stressed by wire mesh restraint for 6h/d/21d or gently handled. During the last 6d of restraint stress, all groups were acclimated to two distinct chamber contexts (A and B) for 10min/day on alternating days. The day following the end of restraint stress and context preexposure, rats were placed in context A and presented with 3x tone-footshock pairings (CS-US; 30s 3tone/0.25mA footshock). The following day, all groups were placed in context B and were presented with 1x CS for memory reactivation and immediately injected with either rapamycin (RAPA, 40mg/kg) or vehicle (VEH, 1% DMSO/5% Tween-80 in saline) and returned to their home colony. Four hours later, rats were placed back into context B and presented with 3x CS to test for post reactivation short term memory (PR-STM). The next day, rats were placed back into context B and again presented with 3x CS to test for post reactivation long term memory (PR-LTM). One week later, rats were placed in context B and presented with 3x CS for spontaneous recovery and two days later, all groups were tested for footshock detection threshold. Two separate subgroups of rats were euthanized immediately after fear memory reactivation (no injection) or PR-LTM for tissue collection and processing for *zif268 in situ* hybridization histochemistry to assess behavior-induced immediate early gene mRNA expression for functional activation.

(A)

Handled Control

Chronic Stress

24h → Conditioning 3x CS-US (Context A) → 24h → Reactivation 1x CS (Context B)

(B) Conditioning

% Freezing to CS

○ CON
■ STR

(C) Reactivation

% Freezing

***

CON   STR

(D) Freezing to Context
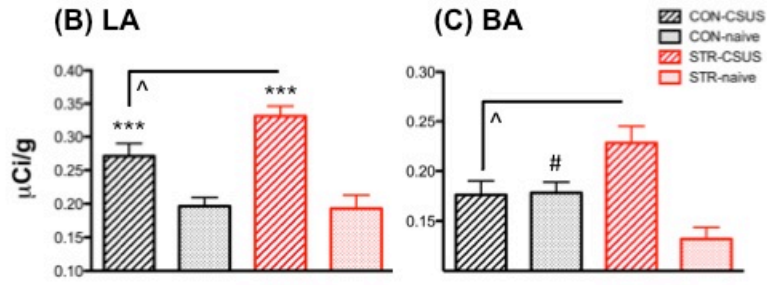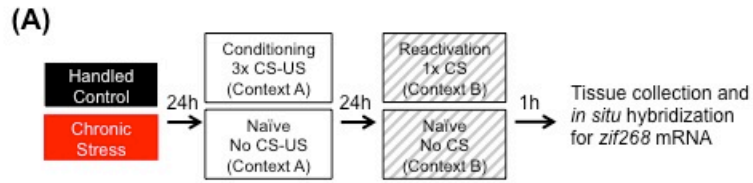
% Freezing to Context

CON   STR

127
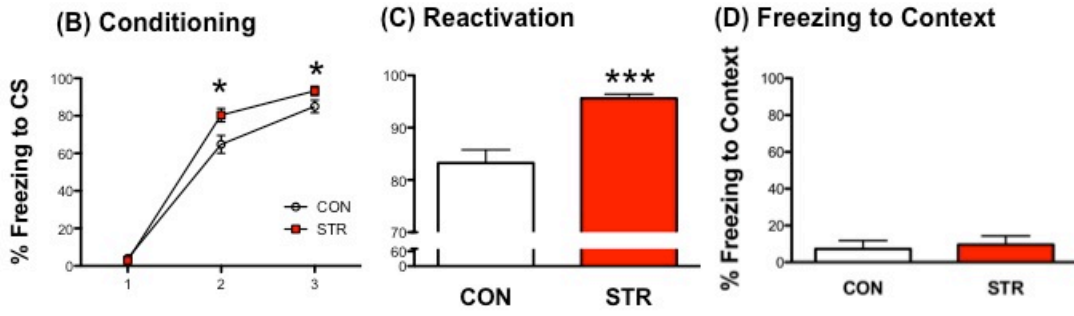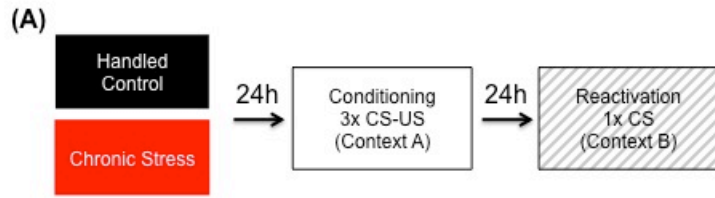
Figure 12. Chronic stress enhanced fear acquisition and memory. Experimental timeline.

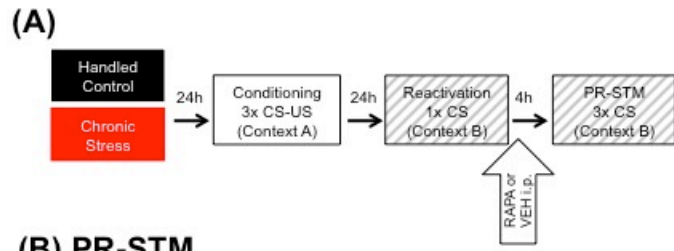(A) Chronic stress enhanced freezing in trials 2 and 3 during fear conditioning. (B)

Chronic stress increased freezing during fear memory reactivation in context B. (C)

Chronic stress did not affect freezing to context prior to the reactivation trial in context B.
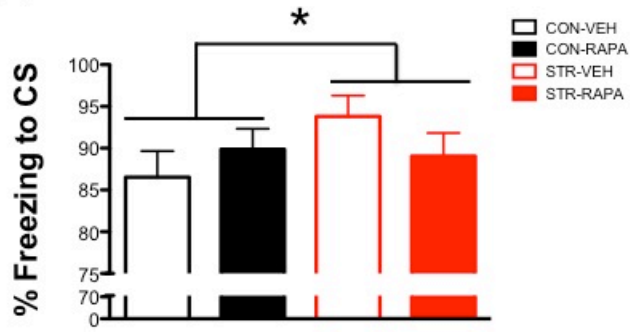
(D) *p<0.05, ***p<0.001 vs. CON. Data are represented as mean ± SEM.

(A)

Handled Control / Chronic Stress → 24h → Conditioning 3x CS-US (Context A) / Naïve No CS-US (Context A) → 24h → Reactivation 1x CS (Context B) / Naïve No CS (Context B) → 1h → Tissue collection and *in situ* hybridization for *zif268* mRNA

(B) LA

μCi/g

(C) BA

Legend: CON-CSUS, CON-naive, STR-CSUS, STR-naive

Figure 13. Chronic stress enhanced amygdala zif268 *mRNA during fear memory reactivation.* Experimental timeline, a subset of rats that were either chronically stressed or gently handled and preexposed to both chamber contexts were either fear conditioned with 3x CS-US or placed in chamber with no stimuli (naïve) to determine chronic stress and fear conditioning effects on *zif268* mRNA induction during fear memory reactivation. (A) Fear conditioning enhanced *zif268* mRNA in the lateral amygdala (LA) during fear memory reactivation compared to naïve groups and planned comparisons revealed that the fear conditioned chronic stress group showed a greater enhancement compared to controls. (B) Chronic stress enhanced *zif268* mRNA in the fear conditioned group compared to controls. There was also greater *zif268* mRNA in nonstressed naïve group compared to the naïve stressed group. (C) Representative autoradiographs of LA and BA *zif268* mRNA during fear memory reactivation following chronic stress and fear conditioning. (D) ***p<0.001 vs. naive; ^p<0.05; #p<0.05 vs. STR-naïve. Data are represented as mean ± SEM.

(A)

Handled Control / Chronic Stress →24h→ Conditioning 3x CS-US (Context A) →24h→ Reactivation 1x CS (Context B) →4h→ PR-STM 3x CS (Context B)

RAPA or VEH i.p.
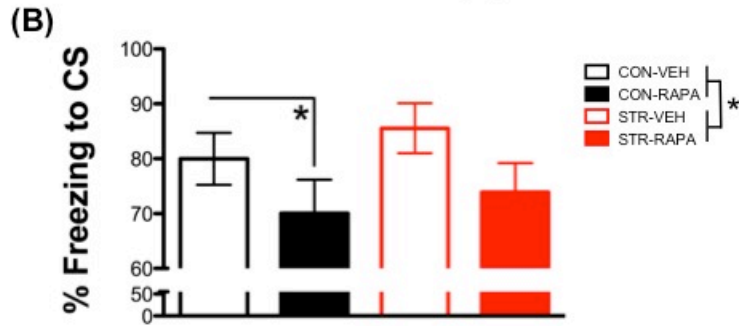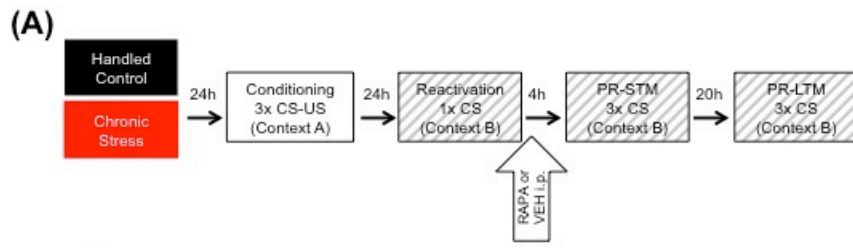
(B) PR-STM

*

% Freezing to CS

CON-VEH
CON-RAPA
STR-VEH
STR-RAPA

*Figure 14. Post retrieval rapamycin did not alter freezing behavior during PR-STM.*

Experimental timeline (A) Chronic stress enhanced freezing to CS during PR-STM, with

no impact of drug. (B) *p<0.05. Data are represented as mean ± SEM.

(A)

| Handled Control / Chronic Stress | 24h | Conditioning 3x CS-US (Context A) | 24h | Reactivation 1x CS (Context B) | 4h | PR-STM 3x CS (Context B) | 20h | PR-LTM 3x CS (Context B) |

RAPA or VEH i.p.

(B)

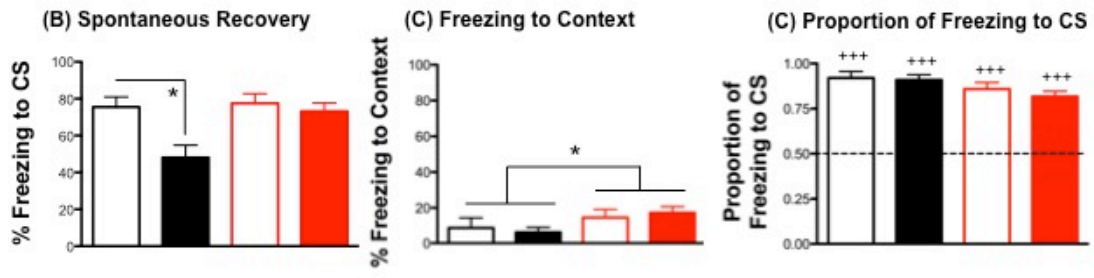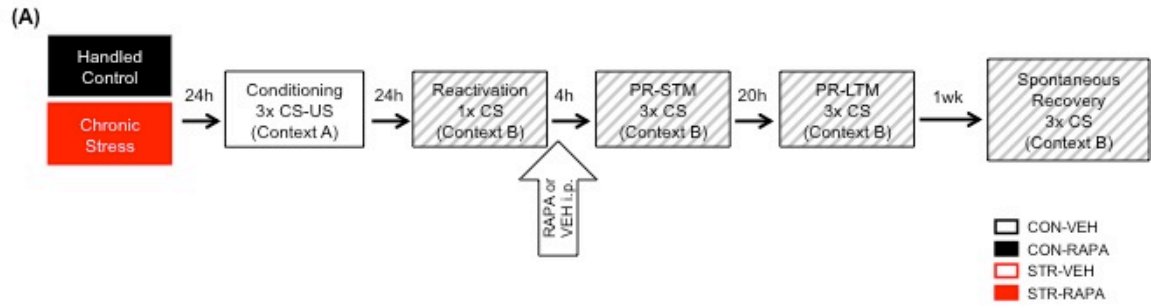*Figure 15. Chronic stress enhanced fear memories are resistant to reconsolidation.*
Experimental timeline (A) 24h after post reactivation rapamycin (RAPA) or vehicle
injection, groups given RAPA showed a general reduction in freezing to CS during PR-
LTM. Planned comparisons revealed a significant decrease in freezing to tone during PR-
LTM within nonstressed controls, while STR-VEH and STR-RAPA were statistically
similar. Chronically stressed groups showed an overall increase in freezing to tone during
PR-LTM. (B) *p<0.05. Data are represented as mean ± SEM.
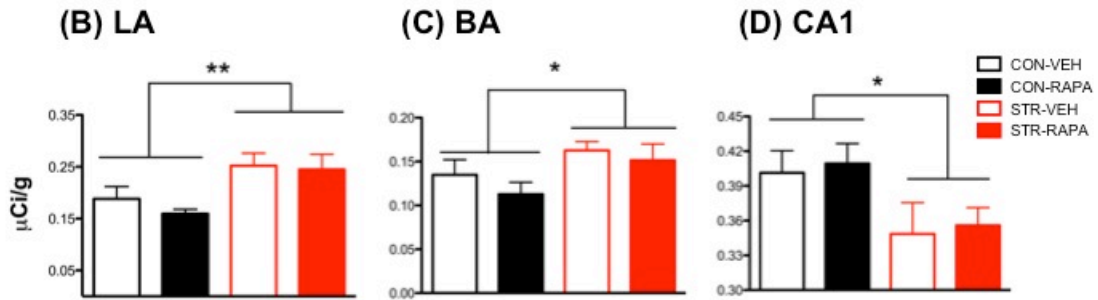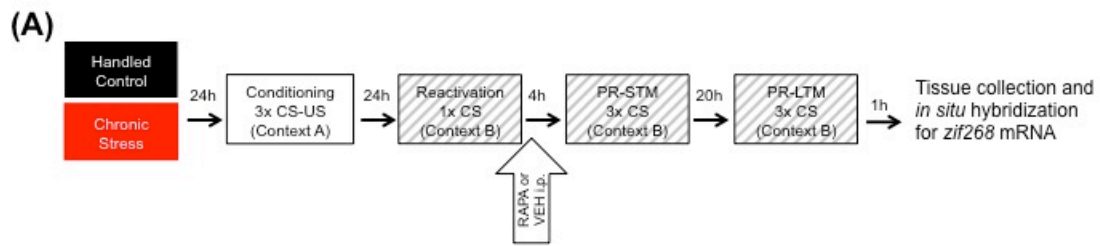
Figure 16. Chronic stress induces contrasting patterns of PR-LTM induced amygdala and hippocampal zif268 *mRNA expression.* Experimental timeline, a subset of groups were euthanized one hour after PR-LTM and brain tissue was processed for *zif268 in situ* hybridization for mRNA expression. (A) Chronically stressed groups showed greater PR-LTM *zif268* mRNA expression within the LA (B) and BA (C) than nonstressed controls. In contrast, these groups showed reduced hippocampal CA1 *zif268* mRNA induction. (D) **p<0.01, *p<0.05; Data are represented as mean ± SEM.
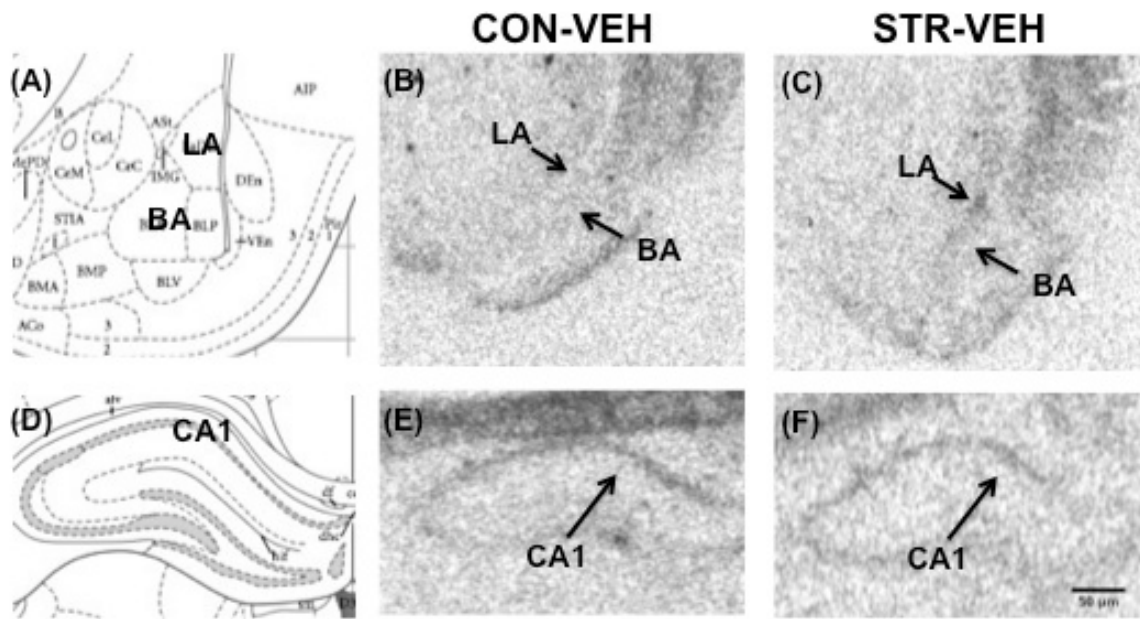
Figure 17. Representative autoradiograph images of chronic stress effects on zif268 *mRNA induction during PR-LTM.* (A and D) Representative sampling areas adapted from Paxinos and Watson (2007) for effects found in LA, BA, and CA1. (B-C) Representative images from CON-VEH and STR-VEH for effects within amygdala subregions LA and BA. (E-F) Representative images from CON-VEH and STR-VEH for effects within amygdala subregions hippocampal CA1.

(A)

Handled Control / Chronic Stress → 24h → Conditioning 3x CS-US (Context A) → 24h → Reactivation 1x CS (Context B) → 4h → PR-STM 3x CS (Context B) → 20h → PR-LTM 3x CS (Context B) → 1wk → Spontaneous Recovery 3x CS (Context B)

RAPA or VEH i.p.

☐ CON-VEH
■ CON-RAPA
☐ STR-VEH
■ STR-RAPA

(B) Spontaneous Recovery

(C) Freezing to Context

(C) Proportion of Freezing to CS

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Figure 18. Effective post retrieval rapamycin effects on blocking reconsolidation are long lasting while associative memory is intact. Experimental timeline (A) Post reactivation rapamycin effects on freezing to CS one week later are still significantly attenuated in nonstressed controls, but not chronically stressed groups. (B) Chronic stressed groups showed low levels, but greater freezing to context B during the spontaneous recovery test when sampled prior to tone onset. (C) All groups showed selective freezing to tone through significantly greater proportion of freezing to CS during spontaneous recovery. (D) *p<0.05, +++p<0.001 vs. 0.5; Data are represented as mean ± SEM.
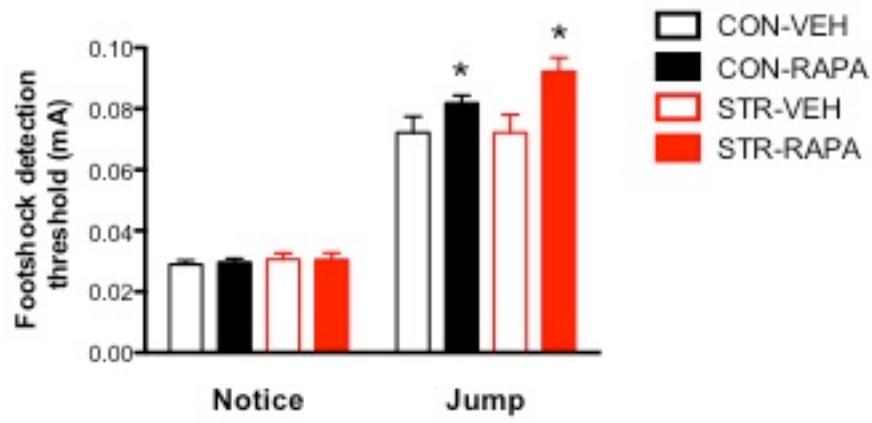
Figure 19. Previous rapamycin increases footshock detection threshold for a jump response. While chronic stress or rapamycin injection had no effect on footshock detection threshold for a notice/orienting response, rapamycin increased footshock threshold detection for a jump response. *p<0.05 vs. VEH; Data are represented as mean ± SEM.
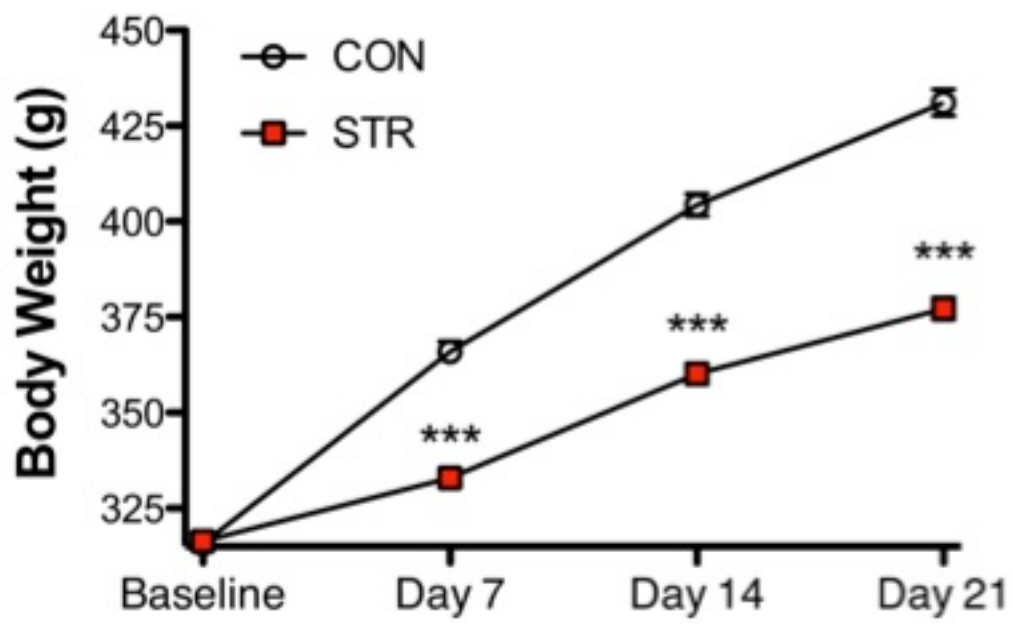
Figure 20. Chronic restraint stress attenuated body weight gain. For all cohorts, chronic restraint significantly attenuated body weight gain across weeks. ***p<0.001 vs. CON; Data are represented as mean ± SEM.

(A) Limbic circuit under baseline conditions

Baseline activity
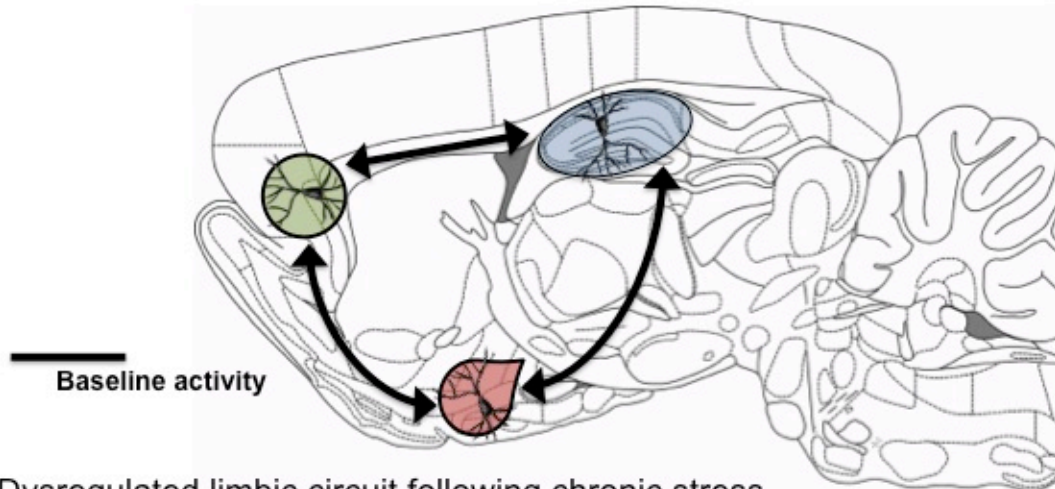
(B) Dysregulated limbic circuit following chronic stress

Hyperexcitability

Increased activity

Decreased activity
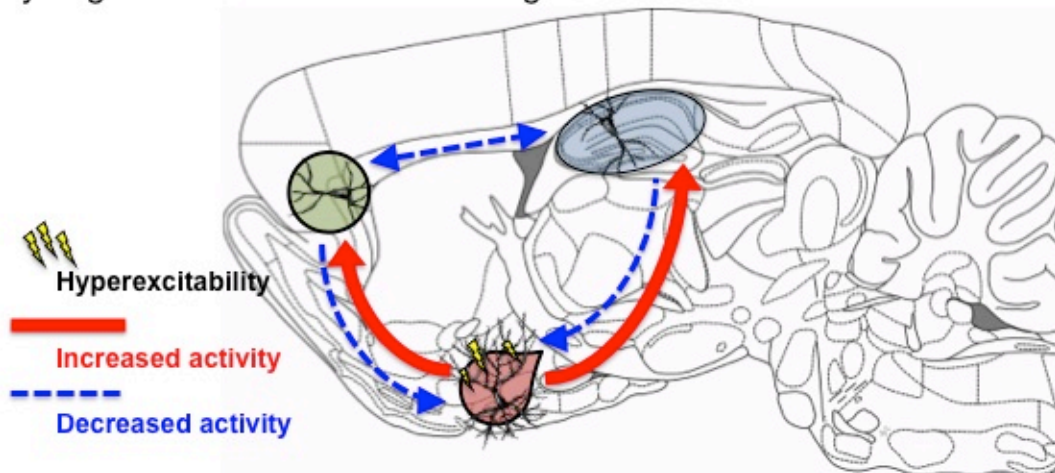
Figure 21. Chronic stress effects on functional plasticity within the hippocampus, mPFC, and amygdala. (A) Schematic sagittal view of a rat brain adapted from Paxinos and Watson (2007) depicting the structural and functional network under healthy conditions (B) Limbic network alterations after chronic stress within the hippocampus, mPFC, and amygdala. These disruptions include structural reduction and hypofunction within the hippocampus and mPFC in tandem with structural enhancement and hyperactivity within the amygdala. This limbic dysregulation is hypothesized to contribute to a vulnerability for PTSD development following exposure to a traumatic event.