EXPOSURE TO ACUTE STRESS INDUCES DIFFERENT PATTERNS OF FEAR EXTINCTION AND HABITUATION BEHAVIOR IN AN ANIMAL MODEL OF POSTTRAUMATIC STRESS DISORDER

A THESIS SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAI‘I AT MĀNOA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF ARTS IN PSYCHOLOGY

DECEMBER 2011

By Michael J. Corley

Thesis Committee:
Lorey K. Takahashi, Chairperson
Robert J. Blanchard
Adrian J. Dunn

Keywords: Stress, Fear, Learning, Memory, Extinction, Sensitization, Habituation, PTSD
ABSTRACT

PTSD is characterized by stress-induced changes that precipitate debilitating behavioral symptoms including indelible fear memories, hypervigilance, and hyperarousal. Current animal models of PTSD do not adequately capture the spectrum of behavioral symptoms. Hence, this thesis examines whether stress-induced conditioned and sensitized fear memories precipitate extinction and habituation resistant PTSD-like fear behavior. Rats were exposed to three intensities (0.0, 0.4, and 0.8 mA) of footshock stress-induced auditory fear conditioning training pairing auditory clicks with cat odor, followed by five consecutive days of extinction testing in a runway with hide box apparatus (Experiment 1). 0.4 mA and 0.8 mA stress-induced fear conditioned rats exhibited extinction resistant fear behavior characterized by sustained increases in hiding, freezing, and head out behavior. This persistent fear behavior is attributed to the retrieval of a stress-enhanced conditioned fear memory because Experiment 2 indicated that withholding the auditory CS presentation during testing did not produce significant effects or elevations in fear behavior. Of further relevance, stress exposure induces a sensitized state, which is a hallmark symptom of PTSD. Hence, to evaluate whether acute stress induces a habituation resistant sensitized fear state, rats were exposed to differing intensities (0.0, 0.4, and 0.8 mA) of acute footshock stress and tested for habituation to a nonassociative auditory click for five consecutive days (Experiment 3). Stress-sensitized rats showed habituation resistant fear behavior characterized by initial enhanced freezing, transitioning into increased head out behavior, and persisting with elevated locomotion behavior. A companion experiment (Experiment 4) revealed rats did not exhibit sensitized fear behavior in the absence of the nonassociative cue. Taken together, results
demonstrate a major role of stress in regulating the persistence of conditioned and sensitized fear memories and provides a relevant model of hallmark PTSD behavioral symptoms.
ACKNOWLEDGMENTS

The author wishes to express sincere appreciation to his adviser Dr. Takahashi for his guidance in developing the experiments to conduct, providing the resources to complete this thesis, and his assistance in the preparation of this manuscript. In addition, special thanks to Dr. Blanchard and Dr. Dunn whose familiarity with the subject matter was helpful during the early planning phase of this undertaking.
# TABLE OF CONTENTS

Abstract i

Acknowledgements iii

List of Figures v

List of Tables vi

Introduction 1

General Methods 12

**Experiment 1**

*Methods* 15

*Results* 16

*Discussion* 21

**Experiment 2**

*Methods* 25

*Results* 25

*Discussion* 29

**Experiment 3**

*Methods* 30

*Results* 31

*Discussion* 35

**Experiment 4**

*Methods* 37

*Results* 37

*Discussion* 41

General Discussion 41

References 44
LIST OF FIGURES

FIGURE 1: Effects of stress-induced auditory fear conditioning on conditioned fear extinction.

FIGURE 2: Effects of stress-induced auditory fear conditioning on habituation.

FIGURE 3: Effects of stress sensitization on habituation to a nonassociative auditory click.

FIGURE 4: Effects of stress sensitization on habituation.
LIST OF TABLES

TABLE 1: Summary of experimental designs.

TABLE 2: Statistical summary of main effects of group, day, and group X day.
INTRODUCTION

Fear is an important emotional state evoked by threat that activates defensive behavior, stress responses, and emotional cognitive processes. Importantly, in a subset of individuals experiencing severe threat they may develop an anxiety disorder known as posttraumatic stress disorder (PTSD), where the threat or traumatic experience is constantly relived through indelible fear memories. Although an integral factor in precipitating PTSD is exposure to extreme traumatic stress, limited knowledge exists about the relationship between stress and the formation of exaggerated and enduring emotional memories. Thus, the scope of this thesis is to understand the integral relationship between acute stress exposure and emotional cognitive processes, which may provide a more comprehensive animal model of PTSD.

Effects of stress on fear behavior.

Stress induces immediate or perceived threat to an individual’s well-being or homeostasis that affects subsequent fear and/or anxiety behavior. Extensive behavioral studies have exposed laboratory animals to various stressors such as footshock (R. J. Blanchard et al., 1968), restraint (Armario et al., 2004), social defeat (Meerlo et al., 1997), and predator (D. C. Blanchard et al., 2003) or predator cues (Takahashi et al., 2005). Electric footshock stress is the most widely characterized aversive stimulus because the intensity, duration, and frequency of footshock stress can be precisely controlled. Additionally, footshock elicits robust behavioral changes such as the freezing response in rats that can be easily quantified. Typically, the procedure involves exposing animals to unavoidable electric shocks through an electric grid. Rats exposed to a single session of footshock stress display increased anxiety behavior in the elevated plus maze.
Mechiel Korte & De Boer, 2003) by spending more time in the closed arms and entering the open arms significantly less than non-stressed rats. Moreover, rats exposed to a single session of footshock show changes in behavior during testing in an open field. Shocked rats show decreased locomotion and rearing; and increased freezing and defecation (Van Dijken et al., 1992). Furthermore, shocked rats tested in an open field with a hide shelter display increased latencies to exit the shelter and decreased locomotion in the arena (Bruijnzeel et al., 2001). The stress-induced behavioral changes are shown to persist for up to 14 days after receiving footshock. More importantly, the effects of acute footshock can last up to 70 days, (Bruijnzeel et al., 2001) indicating that acute stress exposure induces long-lasting behavioral changes. These long-lasting effects of stress on anxiety-like behavior implicates stress as a potential etiological factor in precipitating pathological fear responses common to anxiety disorders such as PTSD.

Ethological approaches to stress have utilized exposure to a predator, such as a cat, (Adamec et al., 2006; D. C. Blanchard et al., 2005) or predator cue, such as cat odor, (D. C. Blanchard et al., 2003; R. J. Blanchard et al., 2001; Dielenberg et al., 2001; Takahashi et al., 2005; Takahashi et al., 2008) as a stressor for laboratory rodents. Rodents exposed to a cat or cat odor exhibit fear-related defensive behavioral responses such as avoidance, flight, freezing, risk assessment, defensive threat, or defensive attack (R. J. Blanchard & Blanchard, 1989a, 1989b; Dielenberg & McGregor, 2001). Rats returned to a chamber or runway apparatus where cat odor was previously experienced show increased hiding and decreased locomotor activity, indicative of contextual fear conditioning (Dielenberg & McGregor, 1999; Takahashi et al., 2008). Additionally, following cat exposure, rats show decreased time and less entries into open arms in the
elevated plus maze, indicating that predator exposure produces prolonged fear behavior (Adamec et al., 1997). Furthermore, these anxiogenic effects are present up to 21 days after cat exposure, (Adamec & Shallow, 1993) supporting similar findings of a long-lasting behavioral change shown with footshock stress exposure.

Taken together, stress exposure profoundly alters an animal’s subsequent behavior dependent on the testing situation and the characteristics of the stressor. Furthermore, these findings raise interesting questions about the persistence of stress-induced changes.

Effects of stress on sensitization and habituation.

Stress exposure evokes one of two memory processes, sensitization or habituation. The first, stress-induced sensitization is a form of nonassociative memory characterized by an increased reaction to a neutral stimulus following exposure to an aversive stimulus (Kandel & Schwartz, 1982). Studies examining stress-induced sensitization usually report a potentiated behavioral response during post-stress testing in a novel context or to the presentation of a neutral stimulus. For instance, rats previously exposed to footshock stress show behavioral immobility to changes in auditory noise (Van Dijken et al., 1992), indicative of a sensitized behavioral response. Furthermore, rats freeze for a significantly higher proportion of time following the administration of a nonassociative auditory stimulus (Murison & Overmier, 1998), suggesting that behavioral immobility/freezing may be the rats’ predominate sensitized behavioral response. However, the broad spectrum of potential sensitized behavioral responses remains not well characterized.
Interestingly, sensitization is hypothesized to inhibit habituation, which is characterized by a decreased response resulting from the repeated presentation of a stimulus (Thompson & Spencer, 1966). In support of this hypothesis, a study using a single 15 min session of footshock stress found persistent high freezing times in rats during habituation to auditory noise over three days (Stam et al., 2002). Thus, acute exposure to footshock stress induced a persistent sensitized behavioral response that was resistant to habituation. The persistent stress-induced sensitized behavior raises further questions about stress inhibiting habituation and may be relevant to the persistent hyper-arousal symptoms characteristic of PTSD.

To further support the notion of a stress-induced sensitized state that is resistant to habituation, predator-based stress appears to be a unique and severe stressor characterized by rapid and long-lasting responses. Rats continue to exhibit anxiety-like behavior after five exposures to cat odor, indicating a resistance to predator stress habituation (Zangrossi Jr & File, 1992). Our laboratory has shown similar results of stressor-induced habituation resistance with repeated exposure to a cat odor cloth in a runway with hide box (Takahashi et al., 2005). The results from this study indicated that rats exposed to an optimal intensity of cat odor over repeated days continued to show avoidance and freezing. Furthermore, another research group reported that while predator stress fear behavior does not habituate in contrast to the hormonal stress response of corticosterone release, which does habituate (File et al., 1993). This finding suggests that habituation is not universal for behavioral and physiological processes. Taken together, these studies highlight the saliency of predator-based stimuli and reveal the distinction between behavioral habituation and neuroendocrine habituation, but these studies only examine
habituation to repeated presentations of the stressor. Thus, it remains to be known if a fear memory acquired from a predator stressor would be resistant to habituation.

**Effects of stress on learning and memory processes.**

Numerous studies reported that stress modulates learning and memory processes (McEwen & Sapolsky, 1995) and often showed that stress impairs cognitive processes. This view arose from the reported learned-helplessness phenomenon in dogs exposed to uncontrollable shock exposure (Overmier & Seligman, 1967). These dogs showed impaired learning during operant conditioning when required to jump across a partition in a shuttle box to escape being shocked. The results of this study suggested that impaired learning may be a result of exposure to stress. However, in contrast to the impairing view of stress, other investigators reported a stress-induced facilitation of classical conditioning using restraint and tail shock stress to enhance classical eye-blink conditioning (Shors et al., 1992). This study provided the first behavioral evidence of stress inducing an enhancing effect on a form of associative memory and suggested that exposure to the stressor induced an altered learning of the stimulus-stimulus association. Furthermore, the stress-induced effect was rapid and persistent. Stress exposure immediately or 24 h prior to eye-blink conditioning training enhanced the eye blink response to the conditioned stimulus (Shors, 2001).

**Effects of stress on fear learning and memory processes.**

Fear conditioning is extensively used to examine emotional learning and memory processes and involves the learned association between a neutral stimulus (the conditioned stimulus, CS), usually context or tone, and an aversive stimulus (the unconditioned stimulus, US), usually footshock. Upon repeated CS-US trials the CS-US
association is learned and presentation of the CS alone elicits conditioned fear behavior known as the conditioned response (CR), most widely assessed with behavioral immobility or freezing (R. J. Blanchard & Blanchard, 1969; Fanselow, 1980). Prior stress exposure potentiates subsequent fear conditioning processes. Similar to Shors’s findings of stress-induced enhancement of classical eye blink conditioning, rats deprived of water showed enhanced contextual freezing to a chamber where shock was received (Maren et al., 1994). Moreover, rats restrained acutely for 2 h and exposed two days later to contextual fear conditioning show enhanced acquisition of conditioned freezing and enhanced recall of conditioned freezing when returned to the context (Cordero et al., 2003). Rats exposed to 21 days of chronic restraint stress show enhanced fear conditioning to both context and tone, suggesting that repeated restraint stress enhances either form of fear conditioning (Conrad et al., 1999). Together, these studies suggest that exposure to either acute or chronic stress prior to auditory or contextual fear conditioning training induces an enhancement of the conditioned fear memory.

More recent work reported that rats administered acute footshock stress prior to contextual or auditory fear conditioning training show enhanced conditioned freezing during the acquisition of conditioned fear memory that can last up to 3 months (Rau et al., 2005; Rau & Fanselow, 2009). Rau proposed a severe pre-shock experience sensitized the rat’s ability to acquire a conditioned fear memory. However, this study makes it difficult to determine the specific effects of shock stress versus the shock-induced contextual fear memory because freezing was the only measure of fear behavior. Thus, an improved approach may utilize an unconditioned stimulus other than footshock for fear conditioning training and analyze a broader spectrum of fear-related behavior.
Effects of stress on the fear extinction process.

Currently, fear extinction is re-examined for its relevance in regulating persistent fear memories characteristic of PTSD (Quirk et al., 2010). The fear extinction process consists of repeated presentations of a non-reinforced conditioned stimulus, which gradually decreases conditioned fear behavior (the conditioned response, CR). Evidence for spontaneous recovery (Brooks & Bouton, 1993; Pavlov, 1927), reinstatement (Pavlov, 1927), and fear renewal (Bouton & Bolles, 1979) supports the notion that extinction is the formation of a new memory and not an erasure of the original conditioned memory. The neural circuitry (Quirk & Mueller, 2008) and facilitation of the extinction process (Quirk et al., 2010) have been extensively examined. However, very few studies have examined the effects of stress on the extinction process or whether a stress-induced fear memory is resistant to extinction. A few reports indicate that mild chronic stress exposure during early life or adulthood impairs extinction of a conditioned fear memory (Garcia et al., 2008; Matsumoto et al., 2008). These studies suggest that a chronic stress-induced fear memory is extinction resistant. To date, an acute stress-induced fear memory has yet to be examined in the context of extinction testing.

Proposed animal model of posttraumatic stress disorder (PTSD).

Currently, ethical limitations of human research prevent invasive methodologies. Therefore, the pathophysiology of PTSD remains largely unknown from clinical studies. Representative and valid behavioral animal models permit an invasive examination of the brain, which will provide a necessary first step in addressing the causal neurobiological underpinnings of PTSD. Moreover, animal models allow pharmacological testing, which may uncover possible targets of therapeutic relief for PTSD. A valid animal model of
PTSD should incorporate exposure to a trauma-like experience and model the behavioral symptomatology, which includes hyperarousal, increased vigilance, and abnormal fear memories (Yamamoto et al., 2009). Yehuda and Antelman provided criteria for a PTSD animal model that include: long-lasting behavioral changes should result from a brief stressor, a spectrum of PTSD-like symptoms should appear dependent on the stressor intensity, and biological changes persist and increase over time (Yehuda & Antelman, 1993). Additionally, distinguishing between associative (fear conditioning) and nonassociative (sensitization) memory-related symptoms is another important component of a PTSD animal model (Siegmund & Wotjak, 2006). The current proposed animal models of PTSD do not satisfactorily address these criteria.

Taken together, this thesis’ experiments attempt comprehensively to address the proposed criteria of a PTSD animal model. First, the model incorporates exposure to an acute stress-induced trauma experience by using stress procedures combined with a predator-based fear-conditioning model. Second, a comprehensive behavioral assessment is used in our model, which captures the spectrum of PTSD relevant vigilance and hyperarousal behavior. Third, conditioned and sensitized fear behavior are examined to distinguish between associative and nonassociative memory-related behavior. Fourth, our model evaluates the persistence of fear memory behavior by observing extinction and habituation over repeated days, which is a hallmark of PTSD’s symptoms. Finally, stressor intensity is examined at different intensities to determine a specific role of stress in enhancing fear memory as well as fear habituation and sensitization.
Summary:

Previous research demonstrated a multitude of stress effects on behavior and cognitive processes including emotional memory. Although these studies are effective at understanding the effects of stress on one day of testing these approaches fail to examine the chronic pathological symptoms found in PTSD. Therefore, the following four experiments examined effects of stress on the acquisition and persistence of fear memories with the overall goal of achieving a more comprehensive animal model of posttraumatic stress disorder (PTSD) that captures the hallmark behavioral symptoms. Future studies can critically examine the neuroanatomical, neurochemical, genetic, and pharmacological underpinnings of the disorder.

Specific Aim 1: Determine the effect of stress-induced associative fear memories on fear extinction and habituation.

Hypothesis 1: Stress-induced auditory fear conditioning will elicit an extinction resistant fear memory.

Experiment 1 Rationale: Evidence for acute stress-induced fear memories effects on fear extinction is sparse. Moreover, the extinction of an auditory fear conditioning memory utilizing predator odor as the unconditioned stimulus has not been examined since most attempts at duplicating fear memories in the laboratory with fear conditioning procedures have relied mainly upon physical stressors such as footshock for an unconditioned stimulus (Fanselow, 1980). Because our laboratory developed predator odor fear conditioning (auditory and contextual) models (Takahashi et al., 2008), we are uniquely able to use footshock as a stressor and predator odor as an unconditioned stimulus for stress-induced auditory fear conditioning. This may provide a unique
approach to distinguish the effects of footshock stress from the conditioned fear memory using an auditory cue associated with predator odor. Furthermore, the rationale for using footshock stress-induced auditory predator odor fear conditioning, which consists of exposing rats to extreme stress and a feeling of life-threatening fear, may model the trauma that precipitates PTSD.

Therefore, Experiment 1 used acute footshock stress exposure of 20 min followed immediately by auditory fear conditioning, pairing auditory clicks with predator odor, and assessed fear behavior over five days of extinction testing in a runway with hide box apparatus to evaluate the effects of stress-induced auditory fear conditioning on fear extinction. Using a runway with box apparatus during extinction testing allows the measurement of freezing in addition to other measures of defensive behavior such as hiding, head out, and locomotion; as well as non-defensive behavior such as lying (D. C. Blanchard et al., 2003; R. J. Blanchard et al., 1986; R. J. Blanchard & Blanchard, 1989a; R. J. Blanchard et al., 2001; McGregor et al., 2005). This spectrum of fear-related behavior may be applicable to modeling PTSD symptomology that includes various components such as avoidance behavior and increased vigilance (Stam, 2007).

**Hypothesis 2**: Stress-induced auditory fear conditioning will induce a habituation resistant fear memory.

**Experiment 2 Rationale**: This study will determine whether stress-induced auditory fear conditioning precipitates an associative and/or nonassociative fear memory that is resistant to habituation. Previous work has shown that habituation does not occur to the repeated presentation of cat odor, but it remains to be known whether a stress-induced fear memory using cat odor as the unconditioned stimulus for fear conditioning
training is resistant to habituation. Furthermore, Experiment 1 may show that a stress-induced auditory fear memory is resistant to fear extinction. Experiment 2 is necessary to suggest this effect results from stress enhancing the conditioned fear memory rather than fear behavior generalizing to another context.

Thus, Experiment 2 was conducted to determine whether generalized fear behavior occurs in the absence of the conditioned stimulus and is resistant to habituation. Specific Aim 2: Determine the effect of stress-induced sensitized fear memories on fear habituation.

Hypothesis 3: Footshock stress-induced sensitization elicits a habituation resistant fear memory that is activated by a nonassociative auditory stimulus.

Experiment 3 Rationale: Footshock stress induces a sensitized fear memory that is resistant to habituation (Stam et al., 2002). The sensitized behavioral response was previously shown to be activated by presentation of a nonassociative cue (Van Dijken et al., 1992). However, in that study only one day of testing was used to examine the sensitized behavioral response and freezing behavior was the only measure.

Therefore, we examined sensitized fear behavior during habituation to a nonassociative auditory click over five consecutive days to determine whether footshock stress-induced sensitization elicits a habituation resistant fear memory that is activated by a nonassociative auditory click. Rats were tested in a runway with hide box apparatus to examine a broad spectrum of potential sensitized fear behavior.

Hypothesis 4: Footshock stress-induced sensitization elicits a generalized habituation resistant fear memory.
Experiment 4 Rationale: This experiment served as a companion to Experiment 3 to assess any generalized effects of stress-sensitization occurring over repeated habituation days. Footshock stressed rats were tested on five consecutive days in the runway with hide box apparatus without the presentation of the nonassociative auditory click.

GENERAL METHODS

Subjects

Naïve male Long-Evans rats (250 - 300 g) bred at the University of Hawaii Animal Facility from a stock obtained from Charles Rivers Laboratory (Raleigh, NC, USA) were single housed in polycarbonate cages with a layer of Bed-o’cobs (The Andersons, Inc., Maumee, OH) and maintained under controlled environmental conditions (21°C, 55% humidity, 12:12 light-dark cycle; lights on at 0600) with food and water provided ad libitum except during testing. All rats were tested between 0800 and 1300 h. Protocols for all experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Animals and approved by the University of Hawaii Institutional Animal Care and Use Committee.

Apparatus

Electric foot-shock box. The shock box (25.3 cm X 20.3 cm X 22.6 cm) consisted of a Plexiglas box with three white sides, a transparent front to allow video recording, and secured top. Scrambled electric footshock was administered via the stainless steel grid floor (San Diego Instruments, San Diego, CA, USA).

Predator-odor auditory fear conditioning box. A clear polycarbonate-housing cage (26.5 cm X 17 cm X 11.5 cm) with the top cover filter insert removed served as the
auditory fear-conditioning box for training. The exposed top of the box was covered by a terry cloth (25 X 25 cm) with or without the presence of cat odor. Fear behavior (freezing) was recorded on digital video for analysis.

*Predator odor cloth.* A new 25 X 25 cm terry cloth was rubbed on the face, neck, armpits, and sides of a spayed male cat and then stored in a sealed plastic bag until its use. All cat odor cloths were handled with disposable nitrile gloves. Our lab has previously shown that rats exposed to a 25 X 25cm terry cloth containing cat odor exhibit high levels of unconditioned fear (Takahashi et al., 2005).

*Runway with hide box apparatus.* The runway with hide box was used for testing fear extinction and habituation. The apparatus (100 cm X 12 cm X 50 cm) consisted of three white Plexiglas sides and a clear Plexiglas front for digital video recording. A white Plexiglas strip 13 cm above the floor projected horizontally 33.3 cm from one end wall to create a low ceiling for a sheltered hiding area. The 100 cm runway box was marked into three equal 33.3 sectors consisting of one covered sector and two exposed sectors.

**Behavioral Measures**

A digital video camera recorded each testing day and the rat’s behavior was analyzed using JWatcher software (Blumstein Lab, Los Angeles, CA, USA) on a laptop computer. The specific behavioral responses that were quantified included: freezing (s) – an immobile alert posture characterized by cessation of body movements except for respiration; head out (no.) – frequency of rat’s head moving and projecting beyond open end of the lowered ceiling while keeping hind legs in the covered hide box portion; hiding (s) – all four paws of the rat were placed in the 33.3 cm section under the low
ceiling area of the runway hide box; lying (s) – the rat’s body and head are in direct contact with the floor; locomotion (no.) – rat ambulation throughout the runway with all four paws across each 33.3 cm floor section.

Statistical Analysis

In all experiments, analysis was based on behavioral data obtained from 8 animals per group (n= 24 per experiment). For the training day, the total duration of freezing exhibited in the conditioning box was converted to percent time and analyzed using a one-way ANOVA procedure. For the five days of extinction or habituation testing (Days 1 to 5) in the runway with hide box, the total duration of freezing, hiding, and lying on each day was converted to percent time and each behavior was analyzed using a 3X5 design repeated measure ANOVA with group (0.0 mA control, 0.4 mA stress, 0.8 mA stress) as the between factor and day (1, 2, 3, 4, 5) as the within factor. The total frequency of head out and locomotion scored on each testing day was also analyzed with a repeated measure ANOVA. If necessary, post hoc analysis was conducted with Tukey’s test.

**EXPERIMENT 1**

**Stress-induced auditory fear conditioning enhances an emotional memory that is resistant to extinction.**

This study tested the hypothesis that exposure to acute stress immediately prior to auditory fear conditioning training will enhance an associative emotional memory that becomes resistant to the extinction of the CS.
Methods: Experiment 1

The experimental design (see Table 1) is described below and consisted of an acclimation day followed the next day with stress-induced auditory fear conditioning training and concluding with 5 consecutive days of auditory fear extinction testing (see Fig. 1A).

Acclimation

All rats were allowed to explore and familiarize to the hide runway apparatus (22 min) and shock box (22 min) in order to minimize the potential elicitation of unconditioned fear responses when subsequently exposed to these apparatuses during training on Day 2 or extinction testing on Day 3. Following acclimation, rats were returned to their home cages.

Stress-induced auditory fear conditioning training

On training day, rats were randomly assigned to one of three footshock stress groups (0.0 mA control, 0.4 mA, 0.8 mA). Control rats in the 0.0 mA group were placed in the shock box for 22 min and not shocked whereas rats in the 0.4 mA and 0.8 mA shock groups were placed in the shock box and after 2 min exposed to one footshock (1s duration) every 2 min for a total of 10 footshocks. Immediately after exposure to footshocks or at the conclusion of the 22 min period, rats in all three groups were transferred to the auditory predator-odor fear-conditioning box for auditory fear conditioning training. Each auditory fear conditioning trial began by covering the top of the conditioning box with a clean cloth for 25 s followed by administering 10 successive auditory clicks (2 msec, 70 db; 1 s apart) with a hand clicker (Gary Wikes Mega Click, J & J Dog Supplies, Galesburg, IL). Midway through the train of 10 clicks, the clean cloth
was switched with the cat odor cloth for 30 s. At the end of this trial, the cat odor cloth was replaced with the clean cloth and another auditory fear conditioning trial commenced. Rats were trained for five auditory fear condition trials, which involved the forward pairing of the conditioned stimulus (CS) clicks with the unconditioned stimulus (US) predator odor cloth. Upon the conclusion of the last trial rats were returned to their home cages until tested the next day for fear extinction.

_Fear extinction testing days_

All groups were tested in the runway with hide box apparatus for five successive days. Each extinction test day began by placing the rat in hide box area of the runway. After 2 min, 2 CS auditory clicks (1 s apart) were administered every 2 min and repeated for a total of 10 presentations of 2 CS auditory clicks. At the conclusion of each extinction test, rats were returned to their home cage. Rat behavior was recorded and scored for the total 22 min duration of each extinction test day.

**Table 1**

Summary of experimental designs.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Acclimation</th>
<th>Footshock Stress (0.0, 0.4, 0.8 mA)</th>
<th>Auditory Fear Conditioning</th>
<th>Testing Days 1 - 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Extinction (CS)</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Habituation (No CS)</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Habituation (Novel Click)</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Habituation (No Click)</td>
</tr>
</tbody>
</table>

**Results: Experiment 1**

_Stress-Induced Auditory-Fear Conditioning Training_

Groups of rats exposed to differing intensities (0.0, 0.4 mA, 0.8 mA) of footshock stress subsequently showed significant differences in freezing behavior during auditory
fear conditioning training, \( F(2,21) = 3.82, p<.05 \) (Fig. 1B.). Post hoc analysis revealed freezing was significantly lower in rats previously exposed to the high intensity (0.8 mA) footshock stress than rats in the no stress (0.0 mA) control group.

**Conditioned Fear Extinction Testing**

Groups of rats exposed to different intensities of footshock stress followed immediately by auditory fear conditioning were tested 24 hrs later over five consecutive days in the runway with hide box for fear extinction by presenting the CS auditory clicks. Repeated measures ANOVA (see Table 2) revealed a significant main group effect for freezing, \( F(2,21) = 5.07; p<.05 \), and head out behavior, \( F(2,21) = 5.56; p<.05 \). Post hoc analysis revealed both 0.4 mA and 0.8 mA stress-induced auditory fear conditioning groups exhibited significantly more conditioned freezing (Fig. 1C) and head out behavior during extinction testing than the no stress (0.0 mA) – auditory fear conditioning control group (Fig. 1D). Further analysis revealed that hiding was elevated (Fig. 1E) and did not significantly differ among groups \( F(2,21) = 1.64; p>.05 \). However, the no stress (0.0 mA) – auditory fear conditioned group exhibited significantly higher lying behavior in the hide box, \( F(2,21) = 18.25; p<.001 \), than the two shock groups (Fig. 1F). The significant group X day interaction for lying, \( F(8,84) = 3.76; p<.001 \), further showed that no stress control rats began to lie in the hide box from extinction test days 2 to 5.

Additional significant group differences were found for locomotion activity, \( F(2,21) = 3.70; p<.05 \) (Fig. 1G). The 0.4 mA stress-induced fear conditioned group displayed more locomotor behavior than the no stress (0.0 mA) – auditory fear conditioned group.

In addition to significant group differences, we found significant day effects for freezing, \( F(4,84) = 13.48; p<.001 \), head out, \( F(4,84) = 2.55; p<.05 \), and lying, \( F(4,84) = \)
Freezing decreased from extinction day 1 to 5, head out increased from 1 to 4, and lying increased from day 1 to 4 and 5, and 2 to 4.

### Table 2
Statistical summary of main effects of group, day, and group X day.

#### Experiment 1

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Group Effect</th>
<th>Day Effect</th>
<th>Group X Day Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freezing</td>
<td>$F(2,21) = 5.07, p&lt;.05$</td>
<td>$F(4,84) = 13.48, p&lt;.001$</td>
<td>$F(8,84) = 1.36, p&gt;.05$</td>
</tr>
<tr>
<td>Head out</td>
<td>$F(2,21) = 5.56, p&lt;.05$</td>
<td>$F(4,84) = 2.55, p&lt;.05$</td>
<td>$F(8,84) = 1.67, p&gt;.05$</td>
</tr>
<tr>
<td>Hiding</td>
<td>$F(2,21) = 1.64, p&gt;.05$</td>
<td>$F(4,84) = 1.01, p&gt;.05$</td>
<td>$F(8,84) = 0.43, p&gt;.05$</td>
</tr>
<tr>
<td>Lying</td>
<td>$F(2,21) = 18.25, p&lt;.001$</td>
<td>$F(4,84) = 7.32, p&lt;.001$</td>
<td>$F(8,84) = 3.76, p&lt;.001$</td>
</tr>
<tr>
<td>Locomotion</td>
<td>$F(2,21) = 3.70, p&lt;.05$</td>
<td>$F(4,84) = 2.49, p&gt;.05$</td>
<td>$F(8,84) = 1.45, p&gt;.05$</td>
</tr>
</tbody>
</table>

#### Experiment 2

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Group Effect</th>
<th>Day Effect</th>
<th>Group X Day Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freezing</td>
<td>$F(2,21) = 2.41, p&gt;.05$</td>
<td>$F(4,84) = 13.06, p&lt;.001$</td>
<td>$F(8,84) = 1.78, p&gt;.05$</td>
</tr>
<tr>
<td>Head out</td>
<td>$F(2,21) = 0.44, p&gt;.05$</td>
<td>$F(4,84) = 1.71, p&gt;.05$</td>
<td>$F(8,84) = 1.65, p&gt;.05$</td>
</tr>
<tr>
<td>Hiding</td>
<td>$F(2,21) = 2.36, p&gt;.05$</td>
<td>$F(4,84) = 1.25, p&gt;.05$</td>
<td>$F(8,84) = 1.74, p&gt;.05$</td>
</tr>
<tr>
<td>Lying</td>
<td>$F(2,21) = 4.50, p&lt;.05$</td>
<td>$F(4,84) = 2.14, p&gt;.05$</td>
<td>$F(8,84) = 0.62, p&gt;.05$</td>
</tr>
<tr>
<td>Locomotion</td>
<td>$F(2,21) = 1.17, p&gt;.05$</td>
<td>$F(4,84) = 0.92, p&gt;.05$</td>
<td>$F(8,84) = 2.83, p&lt;.05$</td>
</tr>
</tbody>
</table>

#### Experiment 3

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Group Effect</th>
<th>Day Effect</th>
<th>Group X Day Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freezing</td>
<td>$F(2,21) = 2.49, p&gt;.05$</td>
<td>$F(4,84) = 64.97, p&lt;.001$</td>
<td>$F(8,84) = 2.95, p&lt;.05$</td>
</tr>
<tr>
<td>Head out</td>
<td>$F(2,21) = 3.24, p&gt;.05$</td>
<td>$F(4,84) = 19.28, p&lt;.001$</td>
<td>$F(8,84) = 2.17, p&lt;.05$</td>
</tr>
<tr>
<td>Hiding</td>
<td>$F(2,21) = 3.84, p&lt;.05$</td>
<td>$F(4,84) = 1.32, p&gt;.05$</td>
<td>$F(8,84) = 1.57, p&gt;.05$</td>
</tr>
<tr>
<td>Lying</td>
<td>$F(2,21) = 4.29, p&lt;.05$</td>
<td>$F(4,84) = 5.61, p&lt;.001$</td>
<td>$F(8,84) = 1.82, p&gt;.05$</td>
</tr>
<tr>
<td>Locomotion</td>
<td>$F(2,21) = 4.89, p&lt;.05$</td>
<td>$F(4,84) = 13.85, p&lt;.001$</td>
<td>$F(8,84) = 4.26, p&lt;.001$</td>
</tr>
</tbody>
</table>

#### Experiment 4

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Group Effect</th>
<th>Day Effect</th>
<th>Group X Day Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freezing</td>
<td>$F(2,21) = 2.39, p&gt;.05$</td>
<td>$F(4,84) = 2.86, p&lt;.05$</td>
<td>$F(8,84) = 1.20, p&gt;.05$</td>
</tr>
<tr>
<td>Head out</td>
<td>$F(2,21) = 4.55, p&lt;.05$</td>
<td>$F(4,84) = 2.43, p&gt;.05$</td>
<td>$F(8,84) = 3.81, p&lt;.001$</td>
</tr>
<tr>
<td>Hiding</td>
<td>$F(2,21) = 0.15, p&gt;.05$</td>
<td>$F(4,84) = 1.14, p&gt;.05$</td>
<td>$F(8,84) = 2.86, p&lt;.05$</td>
</tr>
<tr>
<td>Lying</td>
<td>$F(2,21) = 1.23, p&gt;.05$</td>
<td>$F(4,84) = 2.36, p&gt;.05$</td>
<td>$F(8,84) = 0.87, p&gt;.05$</td>
</tr>
<tr>
<td>Locomotion</td>
<td>$F(2,21) = 0.54, p&gt;.05$</td>
<td>$F(4,84) = 4.24, p&lt;.05$</td>
<td>$F(8,84) = 2.19, p&lt;.05$</td>
</tr>
</tbody>
</table>
Fig. 1.

A

Day 1
Acclimation

Day 2
Shock Stress (0.0 mA, 0.4 mA, 0.8 mA)
+ Auditory Fear Conditioning

Day 3-7
Effects of Auditory CS on Fear Extinction

B

Mean ± SE % Freezing

No Stress Control 0.4mA Stress 0.8mA Stress

C

Mean ± SE % Freezing

Extinction Day

D

Mean ± SE Frequency Head Out

Extinction Day

E

Mean ± SE % IGRT

Extinction Day

F

Mean ± SE Frequency Lying

Extinction Day

G

Mean ± SE Frequency Locomotion

Extinction Day
Figure 1. Effects of stress-induced auditory fear conditioning on conditioned fear extinction. (A) Experimental timeline; rats (n=8 per group) were acclimated, randomly assigned to a stress exposure group (no stress 0.0 mA control, 0.4 mA stress, or 0.8 mA stress), received predator odor auditory-fear conditioning training, and subsequently tested for five consecutive days in the runway with hide box for fear extinction. (B) Mean (+SE) percent freezing behavior during auditory fear conditioning training, *p<.05, 0.8 mA stress group significantly lower than no stress control group. (C) Mean (±SE) percent freezing behavior during fear extinction testing, *p<.05, significantly different from no stress-conditioned control group (D) Mean (±SE) frequency head out behavior during fear extinction testing, *p<.05, significantly different from no stress-conditioned control group. (E) Mean (±SE) percent hiding behavior during fear extinction testing (F) Mean (±SE) percent lying behavior during fear extinction testing, #p<.05, 0.4 mA stress and 0.8 mA stress-conditioned groups significantly different from no stress-conditioned control group on day 2; ##p<.001, 0.4 mA and 0.8 mA stress-conditioned groups significantly different from no stress-conditioned control group on day 3, 4, and 5. (G) Mean (±SE) frequency locomotion behavior during fear extinction testing, *p<.05, 0.4 mA stress-conditioned group significantly different from no stress-conditioned control group.
Discussion: Experiment 1

Extinction of a Stress-Induced Associative Fear Memory

To examine the nature of the relationship of stress-induced associative fear memories on extinction, we addressed in Experiment 1 the hypothesis that acute stress-induced auditory fear conditioning induces an extinction resistant fear memory. Results demonstrated that groups of rats exposed to either 0.4 mA or 0.8 mA stress-induced auditory fear conditioning training displayed significantly higher intensities of conditioned fear behavior over the course of five days of extinction testing, as indicated by increased freezing and head out behavior, compared to control rats that received no stress (0.0 mA) combined with auditory fear conditioning training. Thus, combining footshock stress with predator-odor auditory fear conditioning induces an extinction resistant fear memory characterized by persistent freezing and head out. Taken together, these findings suggest a critical role for acute stress inducing indelible associative fear memories.

The extinction resistant fear memory-related behavior by the stress-induced groups of rats supports the notion of stress modulating learning and memory processes (Bangasser & Shors, 2010; Rodrigues et al., 2009; Shors, 2004) and is consistent with a previous study that reported impaired extinction of a stress-induced conditioned fear memory (Baran et al., 2009). In that study rats were exposed to 21 d of chronic restraint stress prior to auditory fear conditioning training and 24 h later extinction was examined by scoring only conditioned freezing behavior. The authors suggested that chronic restraint stress modulated fear conditioning and affected the recall of the fear extinction memory; however, the persistence of impaired extinction is unknown since only one day
of extinction testing was used. The current study extends these reports by assessing extinction over five consecutive days of testing and quantifying multiple measures of conditioned fear behavior in a complex testing environment, which provides stronger evidence for extinction resistance and reveals additional measures of persistent fear behavior to assess with freezing. Additionally, our study contributes novel information by demonstrating that exposure to acute footshock stress combined with predator odor auditory fear conditioning induces extinction resistant fear.

In contrast, Day 1 extinction results of increased conditioned freezing behavior for stress-induced auditory fear conditioned groups of rats are not consistent with a previous study that reported exposure to acute stress exposure had no effect on auditory fear conditioning in rats (Cordero et al., 2003). A difference between our study and Cordero et al.’s study is that they utilized restraint as the stressor and footshock as the unconditioned stimulus, whereas we used footshock as the stressor and cat odor as the unconditioned stimulus. Thus, restraint stress may specifically enhance contextual fear conditioning in comparison to footshock which may facilitate both auditory and contextual fear conditioning (Shors, 2004). Distinct neural circuits exists for auditory and contextual fear memory processing, supporting the idea that restraint stress affects only contextual fear memory dependent neural regions, such as the hippocampus, whereas footshock stress exposure may affect areas related to both types of fear conditioning (Kim & Fanselow, 1992). Furthermore, footshock and cat odor exposure activate different neural circuits in fear conditioning (D. C. Blanchard et al., 2003; Staples et al., 2005; Takahashi et al., 2008). Taken together, the combination of footshock stress and
auditory fear conditioning with predator odor as the US may elicit an enhanced fear memory that is resistant to extinction.

All groups of rats showed substantially elevated hiding behavior during re-exposure to the auditory click (CS) associated with predator odor over the five days of extinction testing. These results are in agreement with behavioral studies examining the unconditioned defensive behavior profile of rats exposed to predator odor, which report predator odor exposure elicits a suppression of locomotor activity (D. C. Blanchard et al., 2003) and hiding if context permits (Dielenberg & McGregor, 1999). Moreover, our laboratory has previously shown cat odor cloth exposure to elicit persistent hiding behavior during contextual extinction testing in a similar hide runway apparatus (Takahashi et al., 2005). Thus, the results of this study and previous studies are consistent in showing that predator odor exposure increases hiding and reduces locomotion behavior.

Interestingly, during hiding the control rats exposed only to auditory fear conditioning training showed elevated lying behavior starting on extinction day 2 and persisting until day 5 in comparison to both stress-induced groups of rats. Furthermore, these rats showed significantly less freezing and head out across the five days of extinction. Together, these results indicate that with repeated presentation of the conditioned stimulus a non-stress induced emotional memory extinguishes by day 2 and transitions into non-defensive lying behavior. Similar reports of high proportion of time lying were shown in rats not exposed to footshock stress (R. J. Blanchard et al., 1968). Hence, exposure only to predator odor auditory fear conditioning induces a conditioned fear memory that undergoes rapid extinction.
An unexpected result was rats exposed to the high intensity footshock (0.8 mA) exhibited significantly lower freezing behavior, less than 40%, in comparison to the non-stressed (0.0 mA) control group of rats during auditory fear conditioning training in the conditioning box (see Fig. 1B). Without examining conditioned fear memory retrieval on the first day of extinction testing, high intensity acute stress exposure appears to impair the acquisition of the conditioned fear memory. However, the decrease in conditioned freezing during training was unlikely to reflect an impairment in learning. As shown on Day 1 of extinction testing, CS administration increased freezing (Fig. 1C), indicating that a conditioned fear memory was formed and retrieved. Furthermore, freezing was significantly enhanced across the five days of extinction suggesting that stress induced an extinction resistant fear memory.

It should be noted that rats exposed to 0.4 mA stress-induced auditory fear conditioning showed more locomotion activity than the no stress control rats, indicating that these rats were more likely to leave the hide area and explore. These results suggest that 0.4 mA stress may involve a different conditioned fear memory circuitry that motivates locomotor behavior in comparison to no stress control rats.

Taken together, the findings of this experiment have relevance to PTSD since fear conditioning has been suggested to be critical to the development of PTSD (Pitman, 1989) and clinical studies have shown that patients exhibit deficits in the extinction process (Blechert et al., 2007; Milad et al., 2009; Orr et al., 2000; Peri et al., 2000). Hence, our extinction resistant fear memory animal model can be utilized to examine specific neural areas and circuits critically involved in failing to extinguish a fear memory.
Summary:

To summarize, results show that stress-induced auditory fear conditioning elicits persistent increased fear behavior during five days of extinction testing, which is indicative of extinction resistance. Results show acute footshock stress potentiates a conditioned fear memory and suggests a potential behavioral model of extinction resistance, which may serve to examine the pathophysiological correlates of PTSD.

EXPERIMENT 2

Stress-induced auditory fear conditioning does not induce generalized fear that is resistant to habituation.

Conditioned fear behavior was shown to be persistent for rats exposed to stress-induced auditory fear conditioning. Nonetheless, exposure to both predator odor and footshock may induce generalized fear behavior. To examine this possibility, Experiment 2 assessed the generalized fear effects of predator-odor auditory fear conditioning and stress-induced predator odor fear conditioning by omitting the presentation of the auditory conditioned stimulus during habituation testing.

Methods: Experiment 2

In this experiment, we tested the hypothesis that stress-induced fear conditioning will induce an associative / nonassociative fear memory that is resistant to habituation. This hypothesis was assessed using similar testing procedures described in Experiment 1 (Fig. 2A), except during habituation testing in the runway hide box without CS administration (see Table 1).

Results: Experiment 2
Stress-Induced Auditory-Fear Conditioning Training

Groups of rats exposed to differing intensities (0.0, 0.4, or 0.8 mA) of footshock stress showed significant differences in freezing behavior during auditory fear conditioning training, $F(2,21) = 7.09; p<.01$ (Fig. 2B). Freezing was significantly lower for the high intensity (0.8 mA) and intermediate intensity (0.4 mA) footshock stress-induced groups compared to the no stress (0.0 mA) control group ($p<.05$).

Habituation Testing

During habituation testing in the runway with hide box that did not include presentation of the conditioned auditory stimulus, analysis using repeated measures ANOVA (see Table 2) showed no significant main group effects for freezing (Fig. 2C), $F(2,21) = 2.41; p>.05$, head out (Fig. 2D.), $F(2,21) = 0.44; p>.05$, hiding (Fig. 2E), $F(2,21) = 2.36; p>.05$, and locomotion (Fig. 2G.), $F(2,21) = 1.17; p>.05$; however, a significant group effect was present for lying behavior, $F(2,21) = 4.50; p<.05$. Additional analysis revealed rats showed increased hiding behavior and did not differ between groups. However, the no stress (0.0 mA) – auditory fear conditioning control group not only hid but also exhibited significantly more lying behavior than the 0.8 mA stress-induced auditory fear conditioning group (Fig. 2F). Further analysis revealed a significant group X day interaction effect for locomotion behavior, $F(8,84) = 2.83, p<.05$. On day 2 the 0.4 mA stress-induced auditory fear conditioning group was more active than the no stress (0.0 mA) – auditory fear conditioning control group. A significant day effect was found for freezing behavior, $F(4,84) = 13.06; p<.001$. The mean freezing percent for all groups of rats was about 14% on day 1 and decreased to below 5% on days 2 to 5.
Fig. 2.

A

Day 1
Acclimation

Day 2
Shock Stress (0.0 mA, 0.4 mA, 0.8 mA) + Auditory Fear Conditioning

Day 3-7
Effects of Stress-Induced Auditory Fear Conditioning on Fear Habituation

B

C

D

E

F

G

<table>
<thead>
<tr>
<th>Mean ± SE % Freezing</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Stress Control</td>
</tr>
<tr>
<td>0.4mA Stress</td>
</tr>
<tr>
<td>0.8mA Stress</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mean ± SE % Freezing</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Stress + Fear Conditioning</td>
</tr>
<tr>
<td>0.4mA Stress + Fear Conditioning</td>
</tr>
<tr>
<td>0.8mA Stress + Fear Conditioning</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mean ± SE % Hiding</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Stress + Fear Conditioning</td>
</tr>
<tr>
<td>0.4mA Stress + Fear Conditioning</td>
</tr>
<tr>
<td>0.8mA Stress + Fear Conditioning</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mean ± SE % Dying</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Stress + Fear Conditioning</td>
</tr>
<tr>
<td>0.4mA Stress + Fear Conditioning</td>
</tr>
<tr>
<td>0.8mA Stress + Fear Conditioning</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mean ± SE % Locomotion</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Stress + Fear Conditioning</td>
</tr>
<tr>
<td>0.4mA Stress + Fear Conditioning</td>
</tr>
<tr>
<td>0.8mA Stress + Fear Conditioning</td>
</tr>
</tbody>
</table>
Figure 2. Effects of stress-induced auditory fear conditioning on habituation. (A) Experimental timeline; rats (n=8 per group) were acclimated, randomly assigned to an experimental stress group (no stress 0.0 mA control, 0.4 mA stress, or 0.8 mA stress), received predator odor auditory fear conditioning training, and were subsequently tested without presentation of auditory click (conditioned stimulus) for five days in a runway with hide box apparatus. (B) Mean (±SE) percent freezing behavior during auditory fear conditioning training, *p<.05, 0.4 mA and 0.8 mA stress groups significantly different from no stress control group. (C) Mean (±SE) percent freezing behavior during habituation testing. (D) Mean (±SE) frequency head out behavior during habituation testing. (E) Mean (±SE) percent hiding behavior during habituation testing. (F) Mean (±SE) percent lying behavior during habituation testing, *p<.05, no stress-conditioned control group significantly higher than the 0.8 mA stress-conditioned group. (G) Mean (±SE) locomotion behavior during habituation testing, #p<.05, 0.4 mA stress-conditioned group significantly higher than the no stress-conditioned control group on day 2.
Discussion: Experiment 2

Habituation of a stress-induced associative fear memory.

This study examined whether a stress-induced conditioned fear memory using predator odor as the unconditioned stimulus for fear conditioning would be resistant to habituation. Groups of rats exposed to no stress-induced predator odor auditory fear conditioning or stress-induced predator odor auditory fear conditioning did not significantly differ in freezing, head out, hiding, and locomotion behavior when the conditioned stimulus was not presented during habituation testing. These findings suggest that in order to evoke stress-induced conditioned fear behavior, it is necessary to present the conditioned stimulus and activate the recall of the associative fear memory. Moreover, these findings support the results of Experiment 1 showing that persistent conditioned fear behavior resulted from activation of conditioned fear, rather than generalized fear.

Of note, results showed rats exhibited freezing behavior on day 1 of testing (see Fig. 2C). However, freezing decreased rapidly from habituation day 1 to day 2, indicating successful habituation. These findings suggest that fear sensitization (Harris, 1943) or contextual fear generalization (Fanselow, 1980) may influence freezing behavior on the first day of habituation testing.

Consistent with results of Experiment 1, the no stress (0.0 mA) – auditory fear conditioning control group exhibited more lying behavior while hiding in comparison to the 0.8 mA stress – auditory fear conditioning group indicating rats that did not receive footshock were not aroused and more likely to lie down. Furthermore, the 0.4 mA stress – auditory fear conditioned group displayed more locomotor activity on habituation day 2.
in comparison to the no stress (0.0 mA) – auditory fear conditioned control group. Taken together, results suggest exposure to footshock stress may induce a sensitized fear state that affects lying and locomotor behavior, but not freezing and head out behavior.

During predator odor auditory fear conditioning training, rats exposed to either high intensity (0.8 mA) or intermediate intensity (0.4 mA) footshock froze significantly less than rats that received no footshock (0.0 mA) stress. However, in comparison to Experiment 1, results showed the 0.4 mA rats significantly differed in freezing. Individual variability in susceptibility to stress may explain why the 0.4 mA stress group in Experiment 1 was not impaired in freezing during fear conditioning training.

**Summary**

In summary, the current study showed that stress-induced auditory fear conditioning does not elicit generalized fear behavior, which is habituation resistant.

**EXPERIMENT 3**

*Acute Stress Induces Alterations in Sensitized Fear Behavior during Habituation to a Nonassociative Auditory Stimulus*

Experiment 1 suggested that a stress-induced associative fear memory is resistant to extinction. Experiment 2 supported this conclusion by indicating that the persistent conditioned fear behavior was not attributed to fear generalization. Another possibility for increased and persistent fear behavior may be attributed to stress sensitization. Thus, this experiment tested the hypothesis that exposure to acute stress induces a habituation resistant fear memory that is activated by a nonassociative auditory click.
Methods: Experiment 3

Acclimation and habituation testing were identical to Experiment 1, except when rats were exposed to footshock stress they were immediately placed in the conditioning box but not exposed to auditory fear conditioning training (Fig. 3A). Instead, rats were placed in the fear-conditioning box and not exposed to auditory clicks and predator odor. Only a clean cloth was presented.

Results: Experiment 3

No Auditory Fear Conditioning Training

Following the stress exposure condition, groups of rats exposed to clean terry cloths in the conditioning box froze less than 10% and did not differ reliably in freezing duration, $F(2,21) = 0.94; p>.05$ (Fig. 3B).

Habituation Testing During Nonassociative Auditory Click Presentations

Groups of rats exposed to different intensities of footshock stress were tested 24 hr later over five consecutive days in the runway with hide box apparatus for fear habituation during the presentation of nonassociative auditory clicks.

Repeated measures ANOVA revealed a significant group X day interaction effect for freezing behavior, $F(8,84) = 2.95; p<.01$, and head out, $F(8,84) = 2.17; p<.05$. On day 1 of habituation testing during exposure to nonassociative auditory clicks, the 0.8 mA and 0.4 mA stress groups showed more freezing behavior than the no stress (0.0 mA) control group of rats (Fig. 3C). On day 2 and 3 the 0.8 mA stress group showed more head out than the 0.4 mA stress group (Fig. 3D). Further analysis revealed a significant main group effect for hiding, $F(2,21) = 3.84; p<.05$, lying, $F(2,21) = 4.29; p<.05$, and locomotion, $F(2,21) = 4.89; p<.05$, but not head out behavior, $F(2,21) = 3.24, p>.05$. 
Post-hoc analysis showed the no stress (0.0 mA) control group exhibited significantly more hiding (Fig. 3E) and lying (Fig. 3F) than the 0.8 mA stress group. In addition, the 0.8 mA stress group displayed a significantly higher frequency of locomotion in comparison to both the 0.4 mA and no stress (0.0 mA) groups. The significant group X day interaction for locomotion, $F(8,84) = 4.26; p<.001$, further revealed that the 0.8 mA stress group transitioned to increased locomotor activity on day 3, which persisted on habituation day 4 and 5 (Fig. 3G).

Additional analysis showed a significant day effect for freezing, $F(4,84) = 64.97; p<.001$, head out, $F(4,84) = 19.28; p<.001$, lying, $F(4,84) = 5.61; p<.001$, and locomotion, $F(4,84) = 13.85; p<.001$. Freezing decreased from day 1 and 2 to day 3, 4, and 5. Head out increased from day 1, 2, and 3 to day 4 and 5 (Fig. 3D). Lying increased from day 1 to 3 and 5. Locomotion increased from day 1 and 2 to day 3, 4, and 5.
Fig. 3.

A
Day 1
Acclimation

Day 2
Shock Stress (0.8 mA, 0.4 mA, 0.8 mA) (+ No Auditory Fear Conditioning)

Day 3-7
Effects of Stress on Fear Habituation When Presented to Nonassociative Auditory Click

B

C

D

E

F

G

33
Figure 3. Effects of stress sensitization on habituation to a nonassociative auditory click. 
(A) Experimental timeline; rats (n=8 per group) were acclimated, randomly assigned to an experimental stress group (no stress 0.0 mA control, 0.4 mA stress, or 0.8 mA stress) and were subsequently tested for five days of habituation to a nonassociative auditory click in the hide runway apparatus. 
(B) Mean (+SE) percent freezing during presentation of a clean cloth in the conditioning box apparatus. 
(C) Mean (+SE) percent freezing during habitation testing to a nonassociative auditory click, #p<.05, 0.4 mA and 0.8 mA stress groups significantly greater than no stress control group on day 1. 
(D) Mean (+SE) frequency head out during habitation testing, #p<.05, 0.8 mA stress group significantly higher than 0.4 mA stress group on days 2 and 3. 
(E) Mean (+SE) percent hiding behavior during habitation testing, *p<.05, significantly different than the no stress control group. 
(F) Mean (+SE) percent lying during habitation testing, *p<.05, no stress control group significantly different than the 0.8 mA stress group. 
(G) Mean (+SE) frequency locomotion during habitation testing,*p<.05, significantly different than 0.8 mA stress group, #p<.05, 0.8 mA stress group significantly higher than 0.4 mA and no stress control groups on day 3; 0.8 mA stress group significantly higher than no stress control group on day 4, ##p<.001, 0.8 mA stress group significantly higher than 0.4 mA and no stress control groups on day 5.
**Discussion: Experiment 3**

*Stress sensitization effects on habituation to a nonassociative auditory stimulus*

Results showed rats exposed to footshock stress froze significantly more on day 1 of habituation in response to nonassociative auditory clicks than control rats. Furthermore, rats exposed to high intensity (0.8 mA) footshock stress displayed significantly increased head out on habituation day 2 and 3, and significantly increased locomotion on habituation day 3, 4, and 5. These results indicate that high intensity footshock stress induced persistent sensitized fear characterized by initial high amounts of freezing, followed by increased anxiety-like head out behavior, and finally persisting with elevated hyperarousal-like locomotion behavior.

The stress-induced sensitization results support previous findings of footshock stress potentiating freezing in response to a nonassociative stimulus (Murison & Overmier, 1998; Stam et al., 2002; Van Dijken et al., 1992). For example, Stam et al. demonstrated persistent freezing behavior over 3 days of habituation testing. However, our results suggest that high intensity stress induces a persistent hyper-arousal state as evidenced by increased freezing as well as increased head out and locomotion behavior, which is resistant to the habituation of a nonassociative cue over a five-day period. Our novel findings of distinct phases of sensitized fear behavior occurred in our complex testing apparatus, which allowed for a comprehensive analysis of behavior. The use of a runway with hide box allowed rats to hide, freeze, check for threat, or attempt to escape.

In contrast to rats exposed to 0.8 mA stress, the 0.4 mA group of rats did not transition into increased head out or locomotion following initial high amounts of freezing. Moreover, non-stressed control rats displayed minimal locomotion and
increased lying behavior. The results of suppressed lying behavior for stress-induced rats, but increased lying behavior for non-shocked rats is consistent with a previous study revealing that shocked rats do not lie after shock exposure (R. J. Blanchard et al., 1968). Taken together, these results indicate that no stress or exposure to intermediate stress (0.4 mA) does not induce a habituation resistant fear memory and that persistent hyperarousal behavior may only occur after exposure to high intensity stress.

Clinical studies report war veterans exhibit increased behavioral fear responses to the presentation of novel auditory stimuli (Morgan et al., 1996). Thus, the findings of persistent sensitized fear behavior to a nonassociative auditory click may model PTSD’s chronic hyperarousal symptoms.

**Summary**

In summary, high intensity stress induces a sensitized fear memory resistant to habituation when activated by the presentation of a nonassociative auditory cue. Furthermore, results showed habituation resistant sensitized fear is characterized by distinct phases including initial freezing, head out, and persistent locomotion. Furthermore, these results strengthen Experiment 1, which showed that extinction resistant fear behavior reflects a stress-induced conditioned fear memory and not stress-sensitization. Taken together, findings from both experiments provide a method to distinguish between stress-induced associative and nonassociative sensitized fear memories.
EXPERIMENT 4

Acute Stress Sensitization Does Not Induce Spontaneous Habituation Resistance

This experiment examined whether a stress-induced sensitized fear state elicits persistent fear behavior during five consecutive days of habituation testing without presentation of a nonassociative auditory click.

Methods: Experiment 4

As in Experiment 3 (see Table 1), rats were only exposed to footshock stress (0.0, 0.4, or 0.8 mA) on day 2. However, rats were subsequently tested for five habituation days in the runway hide apparatus without presentations of a nonassociative auditory click.

Results: Experiment 4

No Auditory Fear Conditioning Training

Rats previously exposed to different intensities of footshock stress did not differ significantly when exposed to a clean odor cloth in the conditioning box, $F(2,21) = .294; p>.05$ (Fig. 4B).

Habituation Testing

Analysis (see Table 2) showed a significant main group effect for head out behavior, $F(2,21) = 4.55; p<.05$, but groups did not significantly differ in freezing, $F(2,21) = 2.39; p>.05$, hiding (Fig. 4E), $F(2,21) = 0.15; p>.05$, lying (Fig. 4F), $F(2,21) = 1.23; p>.05$, and locomotion, $F(2,21) = .54; p>.05$. Additional analysis of head out behavior revealed a significant group X day interaction effect, $F(8,84) = 3.81; p<.001$ (Fig. 4D). Rats exposed to 0.8 mA stress exhibited more head out behavior than the no stress control group on habituation days 3 to 5 (Fig. 4D).
Further analysis showed a significant day effect for freezing, $F(4,84) = 2.86; \ p<.05$, and locomotion, $F(4,84) = 4.24; \ p<.05$. Freezing was significantly higher on habituation day 2 than day 4 (Fig. 4C) and locomotion was significantly higher on habituation day 1, 2, and 4 than day 5 (Fig. 4G).
Figure 4. Effects of stress sensitization on habituation. (A) Experimental timeline; rats (n=8 per group) were acclimated, randomly assigned to an experimental stress group (no stress 0.0 mA control, 0.4 mA stress, or 0.8 mA stress) and were subsequently tested for five days of habituation in the runway with hide box apparatus without presentation of a novel auditory click. (B) Mean (+SE) percent freezing during presentation of a clean cloth in the conditioning box apparatus. (C) Mean (±SE) percent freezing during habituation testing. (D) Mean (±SE) frequency head out during habituation testing, *p<.05, no stress control significantly lower than 0.8 mA stress group, #p<.05, 0.8 mA stress significantly greater than no stress control group on days 4 and 5, ##p<.05, 0.8 mA stress significantly greater than no stress control group on day 3. (E) Mean (±SE) percent hiding during habituation testing. (F) Mean (±SE) percent lying behavior during habituation testing. (G) Mean (±SE) frequency locomotion during habituation testing.
Discussion: Experiment 4

*Stress sensitization effects on habituation.*

In contrast to the results in Experiment 3, rats previously exposed to differing intensities of footshock stress showed no significant differences for freezing, hiding, locomotion, and lying when a nonassociative auditory click was withheld during five days of habituation testing. Furthermore, when previously stressed rats were immediately placed in the conditioning box but not exposed to fear conditioning training, freezing was reduced and did not differ among groups. Together, these results suggest that a stress-induced sensitized fear state did not spontaneously elicit fear behavior or produce habituation resistant fear.

However, rats in the high intensity footshock group did display significantly more head out on habituation day 3, 4, and 5. These results suggest that high intensity stress induced a generalized sensitized state, which at least elicited increased vigilance-like behavior.

**General Discussion**

Results support the hypotheses that stress-induced associative and nonassociative fear memories affect extinction and habituation. Results of Experiment 1 reported, for the first time, that acute stress-induced predator-odor auditory fear conditioning impairs fear extinction. Furthermore, Experiment 2 ruled out the possibility that persistent fear-memory related behavior across extinction trials was caused by contextual fear generalization. Experiment 3 then showed high intensity footshock stress-induced sensitization to be resistant to habituation when presented to a nonassociative auditory cue. A companion, Experiment 4, demonstrated that footshock stress-induced sensitized
behavior was dependent on the nonassociative auditory cue. Taken together, these results extend previous work examining the effects of stress on the acquisition and extinction of a fear memory and on fear memory sensitization and habituation.

Of relevance to Experiment 1, previous stress research showed glucocorticoids and catecholamines secreted during stress influence learning and memory processes (Rodrigues et al., 2009; Roozendaal et al., 1997). The current results may be attributed to the actions of stress hormones or neurochemicals on the learning circuitry as suggested by Bangasser and Shors (Bangasser & Shors, 2010). Future studies may assess whether our novel stress-induced fear conditioning animal model is mediated by stress hormones or neurochemicals acting on the amygdala, hippocampus, and medial prefrontal cortex to elicit an over-enhanced fear memory resistant to extinction.

Although persistent fear-related behavior did not differ significantly among groups on five consecutive habituation days in Experiment 2 or in Experiment 4, a previous study (McEwen & Gianaros, 2011) showed stress-induced delayed behavioral effects occurring after one week. Since our studies started behavioral testing prior to one-week future work can delay the time between footshock stress-induced exposure and habituation testing to examine whether delayed behavioral effects of stress occur.

Experiment 1 cannot distinguish between the effects of stress on the acquisition and/or consolidation of fear memory. Future research can administer stress at specific post-fear conditioning training time intervals to determine the specific effects of footshock stress on the consolidation process. Previous research reported that the consolidation phase occurs within 3 hrs of post-fear conditioning training (McGaugh, 2000). Therefore, footshock stress exposure can occur immediately or 1 hr after fear
conditioning training to isolate stress effects on the consolidation phase of emotional learning.

Taken together, our animal model has relevance for understanding PTSD. Our model incorporates exposure to a severe acute stressor (i.e. footshock) with a life-and-death trauma-like experience by using predator odor as an unconditioned stimulus for auditory fear conditioning. Our model also addresses both conditioned and sensitized symptoms proposed in a complete PTSD animal model (Mackenzie et al., 2010). Importantly, our PTSD animal model highlights the occurrence of extinction resistant and habituation resistant fear memories and differences in stress-induced associative and nonassociative fear behavior.

In summary, this thesis captures the complex relationship between stress and the modulation of persistent associative and nonassociative fear memories. Future studies examining the underlying mechanisms of stress-induced fear memories may provide novel insight into the pathophysiology of PTSD.
REFERENCES


Blanchard, D. C., Canteras, N. S., Markham, C. M., Pentkowski, N. S., & Blanchard, R. J. (2005). Lesions of structures showing FOS expression to cat presentation: effects on responsivity to a Cat, Cat odor, and nonpredator threat. *Neuroscience & Biobehavioral Reviews, 29*(8), 1243-1253.


Harris, J. D. (1943). *Studies on nonassociative factors inherent in conditioning.* University of Rochester.


of failure to recall extinction memory in posttraumatic stress disorder. *Biological Psychiatry, 66*(12), 1075-1082.


