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The Effect of Chronic Stress on Generalization of Conditioned Fear

College Honors Thesis

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Abstract

Prior lifetime experience of stress is a significant risk factor for posttraumatic stress disorder (PTSD) and other anxiety disorders. The mechanisms by which stress conveys these effects are unknown and likely involve complicated neurobiological alterations. In order to begin to characterize this relationship, we examined the effect of two weeks of chronic stress on fear learning and discrimination. Contrary to our hypothesis, we failed to observe exaggerated fear or poor discrimination in stressed mice. Although the lack of difference in fear learning between stressed and control mice may be attributed to complications in experimental design, these results suggest that the relationship between prior stress and vulnerability to PTSD is more complex than originally conceived.

Introduction

Anxiety and fear disorders (PTSD and phobias) are the most prevalent mental health disorders in society today. Current options for treatment include psychotherapy and pharmacotherapy. Pharmacotherapies are effective for only a subset of those suffering from anxiety and often incur high financial costs, produce adverse side effects, have a negative social stigma, and may result in dependence. Because of this, it is desirable to develop novel treatment options with greater efficacy to ameliorate the detrimental costs of anxiety disorders on society (Sciolino & Holmes, 2012). In order to accomplish this, it is necessary to develop a greater understanding of the contributing causes and neural substrates involved in maladaptive manifestations of anxiety.

Anxiety is often precipitated by stressors in the environment (Padival, Quinette, & Rosenkranz, 2013). These stressors contribute to increased anxiety though potentiation of emotional circuits in the brain (Wood, Norris, Waters, Stoldt, & McEwen, 2008). Stress is known to exert anxiogenic effects on behavioral measures of anxiety and fear learning; it enhances fear learning and changes aspects of emotionality and emotional learning (Buffalari & Grace, 2009; Cohen, Liberzon, & Richter-Levin, 2009; Duits et al., 2015; Reger et al., 2012; Roozendaal, McEwen, & Chattarji, 2009; Suvrathan et al., 2013; Wood et al., 2008;). Increases in anxiety following stress are accompanied by poor fear learning as evidenced by increased generalization of fearful stimuli (Cohen et al., 2009; Reger et al., 2012). In a meta-analysis of fear conditioning in anxiety disorders, Duits et al. (2015) found that individuals with anxiety disorders show impaired discrimination, a tendency to overgeneralize stimuli similar to the CS+. People with

anxiety disorders are unable to inhibit fear in the presence of safety cues and show impaired extinction of conditioned fear (Duits et al., 2015).

Although the experience of a stressful event is inherent in the development of PTSD, not everyone who experiences a traumatic event later develops the disorder. In fact, far more people experience trauma than go on to develop PTSD suggesting that the development of PTSD has more to do with individual differences rather than any specific characteristic of the stressor itself (Keane, Marshall, & Taft, 2006; Lloyd & Turner, 2003). Studies suggest that one factor contributing to the differential vulnerability to anxiety disorders like PTSD is prior experience of stress (Ito, Nagano, Suzuki, & Murakoshi, 2010; Lloyd & Turner, 2003). Lloyd & Turner (2003) found that previous history of lifetime adversity prior to the experience of a serious traumatic event increases risk for developing PTSD following the trauma. Investigations into the relationship between prior experience of stress and vulnerability to PTSD may help to elucidate the mechanisms that underlie the development of this debilitating disorder.

Nonhuman animal models have begun to examine this complicated relationship. Cohen et al. (2009) showed that exposure to extreme stress impairs the ability of the animals to discriminate contextual odor cues in different contexts. This impairment in discrimination produced by stress is dependent on the severity of the stressor; more extreme stress produced a more pronounced inability to contextualize odor cues in a novel context (Cohen et al., 2009). Mild traumatic brain injury, a significant risk factor for the development of anxiety disorders, produces changes in the amygdala and areas related to fear learning (Reger et al., 2012). Following traumatic injury (a form of stress), animals display deficits in fear learning, specifically an increase in context and cued

conditioned fear, as well as overgeneralization of conditioned fear. Stressed animals show increased overall freezing to a tone previously paired with shock when compared with non-stressed animals (Suvrathan et al., 2013).

Despite these advances in our understanding of the relationship between stress and anxiety, there is much nonhuman animal work still to be done in this area. Many studies utilize animal models of PTSD which do little to address the characteristic overgeneralization from CS+ to CS- present in the disorder and often focus on contextual rather than cued fear conditioning (Cohen et al, 2009; Corley, Caruso, & Takahasi, 2012; Hoffman, Lorson, Sanabria, Olive, & Conrad, 2014). It is desirable to develop a model of PTSD that may be manipulated to investigate the effects of chronic stress on generalization, ultimately aiming to examine the cellular and molecular correlates of these effects.

Several areas of the brain have been implicated in the modulation of conditioned fear and are likely involved in the alterations in fear learning following stress. The amygdala is commonly thought to mediate the effects of stress on anxiety and conditioned fear; many of the changes in emotionality and emotional learning produced by stress are dependent on the amygdala (Roozendaal et al., 2009). Individuals with anxiety often display hyper-excitability of the amygdala and increased responsiveness to stress (Duits et al., 2015; Padival et al., 2013). Morphological changes that contribute to this hyper-excitability include increased membrane excitability, increased excitatory synaptic drive, dendritic hypertrophy, and increased numbers of dendritic spines.

The anterior cingulate cortex (ACC) is directly involved in the expression of the fear response and is thought to coordinate predictions of future aversive stimuli and the

appropriate physical behavior (e.g. freezing to tone) (Steenland, Xi, & Zhuo, 2012). Its location between cortical and limbic structures suggests it is involved in the integration of emotion and cognition relevant to fear learning (Bissiere et al., 2008). The ACC is critical to the formation and consolidation of contextual fear memory (Einarsson & Nader, 2012). It shows increased neural activity during fear learning, particularly during tone presentation, suggesting it is critical to the identification of relevant environmental stimuli and the relative danger associated with these stimuli (Steenland et al., 2012). The ACC is thought to exert inhibitory control over the amygdala and other areas involved in the expression of conditioned fear; the assessment of danger and removal of this inhibition results in increased freezing behavior (Steenland et al., 2012). A study by Ito et al. (2010) found a reduction in GABAergic inhibition in the ACC following chronic restraint stress. This suggests that stress may increase excitatory output from the ACC, although Ito et al. (2010) failed to observe increased anxiety accompanying these morphological changes. Stress associated changes in the regions that exert inhibitory control over the amygdala, like the ACC, may be responsible for the enhanced stress reactivity and maladaptive fear response that follows stress.

Prior research in this lab shows that stressed animals display increased freezing to a tone previously paired with shock. This project seeks to investigate the effect of prior stress not only on freezing to the conditioned stimulus, but on discrimination between the conditioned stimulus and a novel stimulus. In order to accomplish this, we first set out to establish a reliable and working model in wild type mice through which to evaluate auditory discrimination between a tone paired with shock and a novel tone. We then subjected naïve mice to two weeks of variable stress prior to testing discrimination. We

hypothesized that two weeks of heterotypic stress would increase fear and poor discrimination. Once established, this model will be used to investigate the neurobiological substrates of stress-associated lack of discrimination.

Method

Experiment 1

Subjects: Eight week old, male C57BL6/J mice (n=8) were housed in groups of 4 and maintained on a 12-hr light/dark cycle with food and water available at all times. Stressor procedures, fear conditioning, and testing all took place between the hours of 11:00 and 15:00.

Stress: Mice were randomly assigned to stressed or no stress groups. Stressed mice were subject to two weeks of variable stress. Each day mice were subject to 1 of 7 different stressors described below:

- Restraint: mice are placed in 250 ml glass beakers for 1 hour
- Swim Stress: mice are placed in a tub of room temperature water and required to swim for 6 minutes. They are then single housed for 30 minutes while they dry.
- Pedestal Stress: mice are placed on a 27 square cm surface elevated 60 cm off the ground for 30 minutes.
- Vibration: mice are placed in a clean show box type cage located on an oscillating table for 30 minutes.
- Foot Shock: mice are given a total of 5 foot shocks. Each shock is 0.6 mA and 2 seconds in duration. (only a subset of mice received this stressor)
- 24 hour continuous room illumination with cage tilt
- 24 hour continuous room darkness with wet bedding

Weighing and Handling: Mice are weighed 5 times over two weeks prior to the fear conditioning procedure (days 1, 4, 7, 10, & 14). Mice undergoing stress are weighed prior to the stressor procedure each weight day. Control mice are weighed, handled and remained in the colony room until undergoing fear conditioning.

Apparatus: Mice are placed in one of two fear conditioning chambers. Each chamber consists of an acrylic box with a stainless steel grid floor through which shock is administered. Each chamber is located within a darkened sound-attenuating chamber equipped with an infrared light and video camera.

Fear Conditioning Procedure: For fear conditioning, the chamber is scented with Vicks Vaporub. After a 2 minute acclimation period, the mouse is given the first of a 5 tone (30 sec, 4KHz, 65 db) and foot shock (.4 mA 1 sec) pairings. 24 hours later, the mice are tested for cued fear conditioning. The chamber is modified to alter the context. The chamber has a solid floor, textured cardboard walls, is lit by a 7W house light and scented with dilute anise extract. After a two-minute acclimation period, mice are presented with a three-minute tone only trial. Freezing in the presence and absence of the tone is quantified using Med Associated Video Freeze Software. Cued fear conditioning is evidenced as greater freezing in the presence versus the absence of the tone.

Experiment 2

Subjects: same as described above (n=31)

Stress: Stressors were altered slightly from the first experiment. Foot shock stress was eliminated to avoid any unintended conditioning to shock prior to fear conditioning. 24 hour light and 24 hour dark stressors were eliminated as they had not been used prior to this experiment in this lab and evidence suggests alterations in circadian rhythm prior to

fear conditioning may adversely impact recall of conditioned fear (Loh et al., 2010). Each day for 14 days mice were subject to 1 of 4 different stressors described below:

- Restraint: mice are placed in 250 ml glass beakers for 1 hour.
- Swim Stress: mice are placed in a tub of room temperature water and required to swim for 6 minutes. They are then single housed for 30 minutes while they dry.
- Pedestal Stress: mice are placed on a 27 square cm surface elevated 60 cm off the ground for 30 minutes.
- Vibration: mice are placed in a clean show box type cage located on an oscillating table for 30 minutes.

Weighing and Handling: same as described above

Apparatus: identical to apparatus described above

Fear Conditioning Procedure: In order to evaluate the effect of stress on discrimination between a tone paired with shock or a novel tone, mice were randomly assigned to tone or noise modalities. Fear conditioning took place in the same context described above. For fear conditioning, the mice assigned to the tone modality receive 5 tone and foot shock pairings; the mice assigned to the noise modality receive 5 noise and foot shock pairings in a similar pattern as the first experiment. 24 hours later and again 48 hours later, the mice are tested for cued fear conditioning in the same altered context described for experiment one. Over the two days following fear conditioning, counterbalanced between stress group and modality, all mice are tested for freezing in the presence and absence of both the tone and noise. Freezing in the presence and absence of the CS+ and CS- is quantified using Med Associated Video Freeze Software. Cued fear conditioning

is evidenced as greater freezing in the presence versus the absence of the auditory stimulus.

Results

Experiment 1

Body Weight (Figure 1): Stressed mice showed attenuated weight gain over the 14 day stress period prior to conditioning ($F(4,6)=11.717$, $p=.000$). No stress animals gained weight.

Conditioning: There were no differences between stressed mice and no stress mice in freezing to the tone during acquisition of fear ($F(4,6)=.558$, $p=.695$).

Testing (Figure 2): Fourteen days of stress did not significantly alter freezing to the tone ($t(6)=.168$, $p=.872$). There was no difference in freezing between stressed and no stress mice.

Figure 1

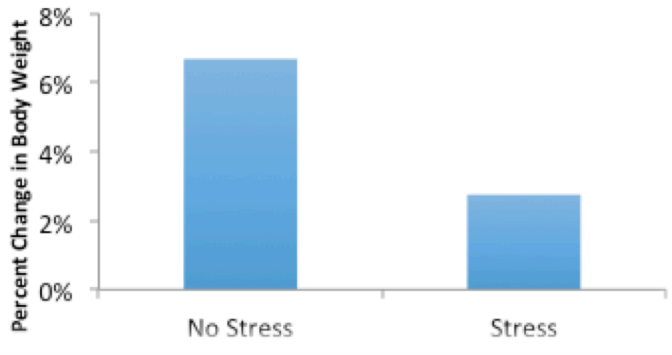
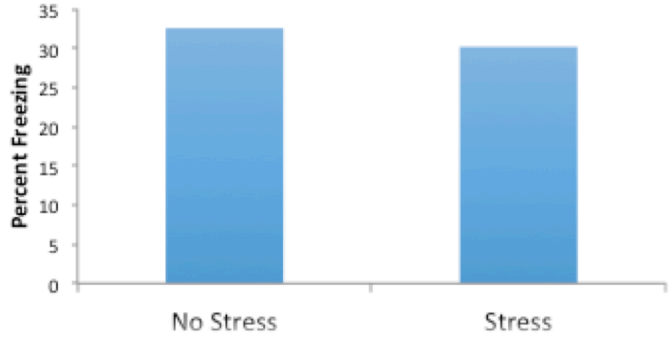


Figure 2



Experiment 2

Body Weight (Figure 3): Stressed mice showed attenuated weight gain over the 14 day stress period prior to conditioning ($F(4,27)=9.93$, $p<.01$). No stress animals gained weight.

Conditioning: There was no difference between stressed mice and no stress mice in freezing to the CS+ during acquisition ($F(4,27)=.979$, $p=.422$). There was also no difference in freezing to the tone or noise CS+ during acquisition. ($F(4,27)=.933$,$p=.448$).

Testing (Figure 4): Overall, mice discriminated between the CS+ and the CS-, evidenced in greater freezing to the CS+ than to the neutral CS- ($F(1,27)=76.292$, $p=.000$). There was no difference in discrimination between mice that were stressed and those that were not stressed ($F(1,27)=2.926$, $p=.099$). However, the interaction approached significance, and interestingly, contrary to hypothesis, the data suggest that mice that were stressed showed better discrimination (less generalization).

Figure 3

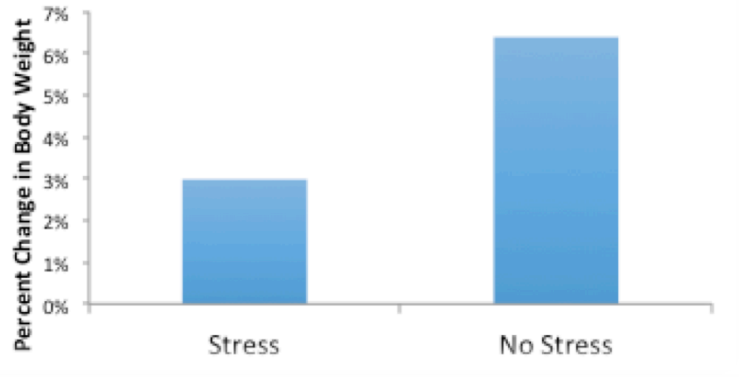
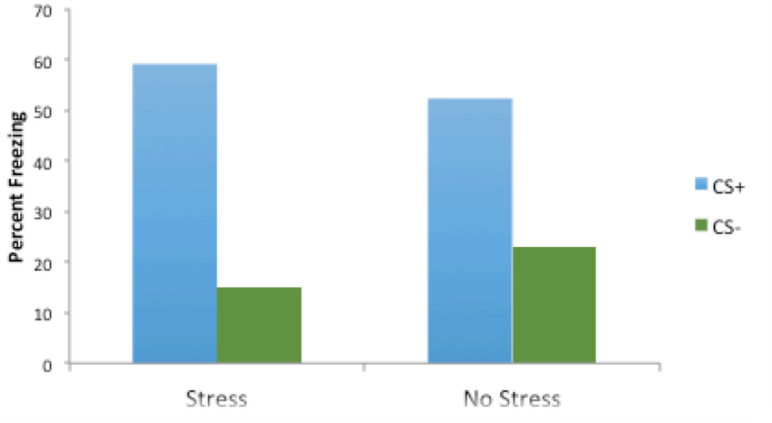


Figure 4



Discussion

These experiments investigated the effect of two weeks of heterotypic stress on discrimination between a stimulus paired with shock (CS+) and a neutral stimulus (CS-). Previous research in this area has demonstrated that stress tends to increase fear to a CS+ (Falls, Fox, & MacAulay, 2010). However, contrary to hypothesis, we failed to observe an increase in freezing, evidence of exaggerated conditioned fear, among stressed mice. We did observe discrimination between CS+ and CS- among both stressed and no stress mice, although discrimination was not significantly different between the two groups. We failed to observe weakened discrimination following stress. In fact, although the result only approached significance, the data suggests that stressed mice actually showed better discrimination between the CS+ and CS-.

Exposure to chronic stress increases anxiety and fear behaviors (Hammock et al., 2009). Previous research in our lab exposed mice to two weeks of heterotypic stress and observed exaggerated fear as measured by increased acoustic startle amplitude. This observation was an important starting point for the theoretical framework of this experiment. Contrary to our expectations, we failed to see this effect; there are several minor differences between our pilot studies where we observed exaggerated conditioned fear in stressed mice and the experiments discussed here where we failed to replicate this effect.

The mice used in this experiment were group housed, although previous experiments in this lab often single housed mice. Environmental factors such as environmental enrichment or social support can confer stress resilience in the face of trauma (Lupien, McEwen, Gunnar, & Heim, 2009). In fact, perceived social support is

strongly associated with resilience following trauma (Keane et al., 2006). Unpublished data from our lab shows that 2 weeks of heterotypic stress in combination with single housing produces the most robust stress effects, rather than either alone. It is possible that our failure to observe exaggerated conditioned fear in stressed mice was a result of the protective effects of social support that may result from group housing.

Despite not single housing mice, we did observe an effect of stress on body weight suggesting that our stressors were effective at some level. A study by Martí, Martí & Armario (1994) showed that the severity and duration of stressors is proportional to effects on weight gain. To ensure that the stressors used are producing other changes, it would be valuable to take another physiological measure of stress, such as blood or saliva stress hormone levels, in addition to body weight. Although differences in body weight are a reliable marker of stress effects, they do not allow us to examine more discretely timed changes in neurochemistry that are likely responsible for some of the effects of stress on fear and anxiety. The collection of this additional body correlate of stress will allow for a more accurate gauge on specific alterations in the stress response, which varies both throughout the day and lifespan (Lupien et al., 2009).

Although the experience of stress did result in changes in body weight, it is possible that the level of stress produced by the stressors used during this experiment may not have been intense enough to cause changes in conditioned fear. The first experiment utilized stressors new to our lab and results may be complicated by the inclusion of foot shock and alterations in circadian rhythm prior to fear conditioning. In order to address this, fewer stressors were used in experiment two than previous experiments in this lab. Although this was done to avoid confounding factors in our analysis, it is possible that

this change attenuated the intensity of the stressors. Responses to stress are more robust when the stressor is varied each day; it is likely that fewer stressors may have resulted in greater habituation to the stressors used and a less intense experience of stress. More predictable, milder stress has been associated with enhancements in memory function (Parihar, Hattiangady, Kuruba, Shuau, & Shetty, 2011)—it may be that this less intense experience of stress produced beneficial effects on fear memory and enhanced discrimination in stressed mice. Replication of this experiment with the addition of other stressors could reduce any potential habituation that may occur when presented with the same stressor multiple times.

Failure to observe an effect of stress on fear conditioning may also be the result of a ceiling effect. The level of fear conditioning observed in control mice was slightly higher than what is typically observed in our lab. It is possible that measurements of freezing from both controls and stressed mice were exceptionally high, masking any potential effect of stress on fear conditioning. This experiment used animals housed in a large, busy colony room; previous experiments performed in this lab used animals housed in a smaller, less trafficked colony room closer in proximity to our lab. It is possible that this change in colony room may have resulted in baseline stress to all of the animals used in this experiment, producing generally higher levels of freezing observed in control and stress mice. In order to investigate this possibility, it will be interesting to replicate this experiment using animals located in a smaller colony room.

The diverse and varied characteristics of "stress" in the human experience call into question the ecological validity of the stressor procedures used in this study. As not all stress is qualitatively the same, it follows that different types of "stress" might

produce different physiological effects. A study looking at the effects of post-natal stress on glucocorticoid functioning found that maternal deprivation produces effects opposite to those produced by severe abuse in childhood (Lupien et al., 2009). It has also been documented that exposure to mild, predictable stress can be beneficial to brain function, specifically enhanced memory (Parihar et al., 2011). These seemingly contradictory effects of stress on physiological functioning combined with the difficulty in translating conceptualizations of stress between human and rodent models highly complicates the ability to create an easily manipulated stressor procedure in rodents while maintaining ecological validity.

Although the etiology of PTSD is straightforward in that there is a single, discrete traumatic event responsible for the development of the disorder, study of and attempts to model the disorder are complicated by the fact that, unlike simple phobias, re-experiencing symptoms that characterize the disorder are often cued by situations or emotions that closely resemble or symbolize the original trauma. Rather than an identifiable, tangible signal, discrimination between a secure situation and one in which there is cause for heightened fear in an individual with PTSD may often be subjective and difficult to trace to one specific cause. Since overgeneralization in PTSD can be the result of extremely human, personal, cues such as moods or even anniversaries of the trauma (Keane et al., 2006), there may be no satisfactory way to fully model the disorder in rodents.

Although we did not find that stress negatively impacted discrimination, it is well documented in human literature that prior experience of stress is a significant risk factor for PTSD (Keane et al., 2006, Lloyd & Turner, 2003, Lupien et al., 2009). It is likely that

we failed to observe an effect of stress on discrimination because the link between prior stress and risk for PTSD is more subtle and complex than originally conceived in the theoretical framework of this experiment. Prior experience of stress may not directly impact discrimination. Research has shown that individual variability in response to stress in humans is highly dependent on the person's appraisal of the stressor. An individual's tendency to "catastrophize" negative events is strongly correlated with increased risk for developing PTSD (Keane et al., 2006). Emotion regulation, learned coping strategies, and the appraisal of a stressor as a disaster versus a challenge are all complex elements contributing to the expression of the human stress response that are difficult to measure and quantify in research. Additionally, stress may encourage maladaptive behaviors in humans such as drug abuse, poor sleep, and poor nutrition that contribute indirectly to inefficient processing of traumatic experiences and ultimately could result in poor discrimination.

Despite these complications, the development of this model is still useful to the understanding of the etiology of anxiety disorders like PTSD. Once established, this model will be used to further investigate the effects of prior stress on fear and anxiety. Studies have shown that the risk for PTSD increases when mild, general stress is experienced following a traumatic event (Lloyd & Turner, 2003). It may be interesting to compare the effect of stress prior to fear conditioning with the effect of stress following fear conditioning and prior to testing. In addition, the age at which stress is experienced and the type/severity of stress undergone likely differentially impacts the risk for development of PTSD and anxiety disorders. It will be valuable to our understanding of the etiology of anxiety disorders to vary these qualities within this framework to

investigate these relationships. This model will ultimately be a useful tool with which to study the neurobiological correlates of the effects of stress on discrimination and fear learning. Specifically, future studies will seek to characterize the role of the amygdala, anterior cingulate cortex and related areas in the maladaptive effects of stress on fear and anxiety.

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