THE ROLE OF CORTICOSTERONE AND IL-1β ON FEAR MEMORY

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by

Adam C. Kulp

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Dissertation written by

Adam C. Kulp

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Ph.D., Kent State University, 2019

Approved by

_____, Chair, Doctoral Dissertation Committee
John D. Johnson, Ph.D.

_____, Members, Doctoral Dissertation Committee
Colleen B. Novak, Ph.D.

_____
Aaron Jasnow, Ph.D.

_____
Richard S. Meindl, Ph.D.

_____
Gary K. Koski, Ph.D.

Accepted by

_____, Director, School of Biomedical Sciences
Dr. Ernest J. Freeman, Ph.D.

_____, Dean, College of Arts and Sciences
Dr. James L. Blank, Ph.D.

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Chapter 1

Literature Overview

Clinical Relevance

Anxiety is a broad diagnostic category that ranges, but is not limited to, generalized anxiety disorder, panic disorder, social anxiety disorder, and post-traumatic stress disorder (PTSD). An underlying behavioral characteristic in all these disorders is an enhanced fear response, more specifically, an enhanced fear memory. Normally, this is evolutionarily beneficial since the organism will remember the stressful events and avoid them in the future. Unfortunately, this behavior can become damaging if the memory causes more stress than the event itself (anxiety disorder). In turn, this leads to a cascade of other clinical issues. For example, insomnia, a sleep disorder in which patients have difficulty sleeping, is one of the clinical conditions that has a high comorbidity with anxiety (Buckner *et al*., 2008). Most notably, this occurs when patients have late night thoughts of the stressful memories of their past. Moreover, the patient has a disruptive awake/sleep cycle (Staner, 2003). Overall, this dilemma leads to $63.2 billion loss in American workforce production per year (Kessler *et al*., 2011). As

only one example of how anxious memories can induce clinical issues, the literature suggests more research is needed to prevent and treat anxiety.

Kessler *et al*. is one of the leading teams that investigates the prevalence of clinical issues. In particular, their work in 2005 measured the severity, prevalence, and comorbidity of different forms of anxiety and emotional disorders. To accomplish this, they asked adults to take the World Health Organization World Mental Health Survey, which would indicate whether an individual has an anxiety disorder or other clinical issues. Not surprisingly, Kessler *et al*. discovered a large portion of individuals have a form of anxiety (18.1%) and have high comorbidity with other behavior disorders (2005). Interestingly, there is a large disparity in the prevalence rates of anxiety between women and men: women 23.4% and men 14.3% (Kessler *et al*., 2005). Moreover, women are twice as likely to develop PTSD (Foa and Street, 2001). Vesga-Lopez *et al*. investigated the disparity in anxiety between men and women by utilizing the National Epidemiological Survey on Alcohol and Related Conditions. The results suggest that women are more likely to have comorbidity with other mood and anxiety disorders. On the other hand, men are more likely to use alcohol and non-prescription medications to relieve anxiety (Vesga-Lopez *et al*., 2008). In addition, Vesga-Lopez *et al*. (2008) discovered that women are more likely to develop anxiety behaviors if they have a family history of anxiety compared to men. Last, Vesga-Lopez *et al*. (2008) investigated whether the rate of treatment seeking was responsible for prevalence of sex differences. The data revealed that both sexes had a low treatment-seeking tendency; however, women were more likely to find treatment (Vesga-Lopez *et al*., 2008). It is still unclear what, at the molecular level, is causing the differences in susceptibility between women and men. One possibility is differences in the regulation of the basolateral amygdala (BLA), a brain region that contributes to the formation of fear memories,

and a brain area where female rats show greater synaptic excitability compared to males (Blume *et al*., 2017).

Basolateral Amygdala (BLA)

The formation of fear memories involves the following major brain regions: amygdala, hippocampus, bed nucleus of stria terminalis, nucleus accumbens, entorhinal cortex, hypothalamus, auditory cortex, and parabrachial nucleus (Janak and Tye, 2015). The BLA, a subregion of the amygdala, is particularly important in making associative memories. The associative phenomenon was demonstrated in Maren's work through lesioning of the BLA (1999). In particular, Maren was able to show that lesioning the BLA following fear conditioning prevented rats from remembering that an arena is dangerous following the initial fear conditioning (Maren, 1999). The BLA utilizes glutamatergic neurons and inhibitory interneurons to regulate the fear memory circuit (Janak and Tye, 2015). The circuit starts when a stimulus affects the organism (in the case of contextual fear conditioning animals are exposed to a novel conditioning chamber and a series of electric foot shocks). The paraventricular subregion of the dorsal midline thalamus receives this signal and then projects to the lateral amygdala (Do-Monte, Quinõnes-Laracuente, & Quirk, 2015). Following, the lateral amygdala projects to the BLA, which then sends signals to the central amygdala, which sends signals to periaqueductal gray, an important brain region for freezing behavior. In addition, the BLA will send signals to the prefrontal cortex and hippocampus (Janak and Tye, 2015). The hippocampus also projects to the BLA to relay the context (environmental cues; Kishi *et al*., 2006), and it is here, within the BLA, that context gets associated with an aversive stimulus (Figure 1). The BLA is the location of focus in these studies since during high stress the locus coeruleus stimulates the BLA (Giustino

and Maren, 2018), and the BLA regulates the associative memory between aversive stimuli and the context (Maren, 1999; Gale et al., 2004; Phillips and LeDoux, 1992). In turn, it makes it a valuable region to study for anxiety and stressful behaviors.

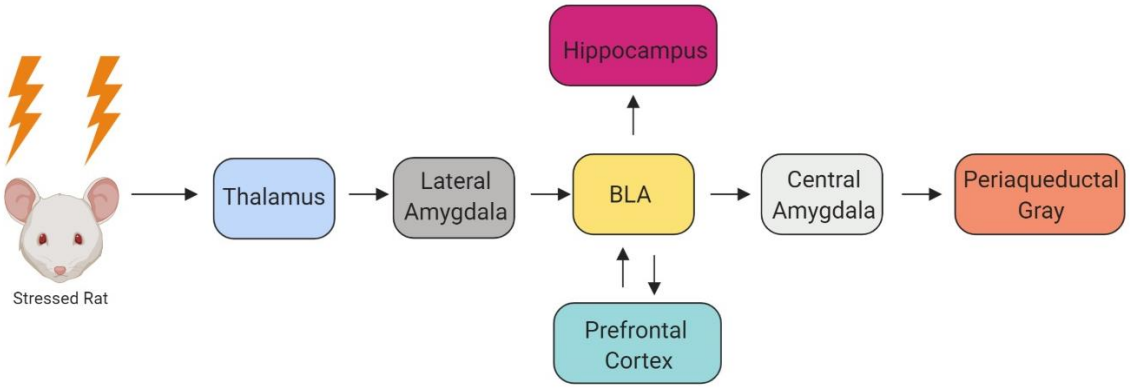

Figure 1. Circuitry of a Fear Memory: The circuit starts when a stimulus affects the organism (in the case of our fear conditioning paradigm the aversive stimulus is a series of electric foot shocks). The thalamus receives this signal and then projects to the lateral amygdala. Following, the lateral amygdala projects to the BLA, which then sends signals to the central amygdala, which sends signals to periaqueductal gray, an important brain region for freezing behavior. In addition, the BLA will send signals to the prefrontal cortex (bidirectional) and hippocampus (Janak and Tye, 2015). The hippocampus also projects to the BLA to relay the context (Kishi *et al*., 2006), and it is here, within the BLA, that context gets associated with an aversive stimulus.

Stress and Anxiety

Men and women respond to stress differently. Interestingly, there are many studies that find CORT is lower in women compared to men (Zimmer *et al*., 2003; Van Cauter *et al*., 1996; Seeman *et al*., 2001; Zhao *et al*., 2003; Kajantie and Phillips, 2006; Kudielka and Kirschbaum, 2005; Kumsta *et al*., 2007; Schoofs and Wolf, 2011). However, there are other articles that find no differences between men and women (Kelly *et al*., 2008; Wiemers *et al*., 2013). In a review article by Verma *et al*., they describe the difference as 'Fight or Flight vs Tend and Befriend model' (2011). The models require both the hypothalamus pituitary adrenal (HPA) axis and sympathetic nervous system; however, they are utilized differently depending on biological sex. Specifically, during a stressful event, men will fight or run from the event. Moreover, women utilize more limbic brain regions: putamen — insula — ventral striatum — and cingulate cortex during times of stress (McClure *et al*., 2004). The sex differences in brain activation could contribute to the differences in anxiety. Fear memory, which occurs when a stressful event forms an emotional memory, is thought to be particularly important in triggering anxiety. A fear memory requires many brain regions, but the major regions include: the hippocampus, amygdala, and medial prefrontal cortex (Lebron-Milad *et al*., 2012). Lebron-Milad *et al*. wanted to determine whether these brain regions were sexually dimorphic when given an aversive memory in humans (2012). They had participants go through functional magnetic resonance imaging during fear conditioning and extinction. Interestingly, women had greater signaling in the amygdala compared to men (Lebron-Milad *et al*., 2012). Further information is needed clinically on whether chronic stress prior to fear conditioning enhances these activations.

The rodent literature has similar findings compared to clinical work, but the rodent literature can provide more insight into the molecular mechanisms behind anxiety-like behaviors.

There are many tests to measure anxiety-like behavior: open field — elevated T-maze — light/dark — passive avoidance — and all the listed tests have sexual dimorphic differences (Kokras and Dalla, 2014; Chang *et al*., 2009). Interestingly, the literature also suggests that stress modulates anxiety-like behaviors in these tests (Matuszewich *et al*., 2007; Lezak, Missig, and Carlezon, 2017; Ihne *et al*., 2012; Camp and Johnson, 2015). As an example, José *et al*. chronically stressed male rats and found that the chronic stress sensitized anxiety-like responses in the plus maze test (2016). Our laboratory was interested in this phenomenon and adapting it to contextual fear memory. Specifically, we investigated the neuroendocrine response that facilitates fear memory following chronic stress, particularly, corticosterone and catecholamine response.

The Role of CORT on Fear Memory

Cortisol (corticosterone in rats or CORT), a hormone that is produced in response to stressful stimuli, is a potential candidate molecule for enhanced fear memories and other anxiety-like behaviors. Currently, there is a disagreement within the literature on the molecular mechanism for the sex differences in anxiety. Understanding the potential role of CORT in promoting anxiety behavior is valuable for preventing and treating anxiety.

CORT is a glucocorticoid that is produced via the adrenal glands. Pugh *et al*. was one of the first laboratories to work on CORT's role in fear memory, specifically through adrenalectomies (1997). Pugh *et al*. investigated whether CORT contributes to contextual fear memory by removing the adrenal glands from rats. After recovery, rats underwent contextual fear conditioning, which was a single 2 sec foot shock (0.4mA). After 24 hours following conditioning, rats were placed in the same context and percent freezing (contextual fear memory)

was measured. As expected, the adrenalectomized rats had a significant decrease in their percent freezing. Pugh *et al*. continued this work to try to rescue the behavior. This was accomplished by adding CORT into their drinking water. Unsurprisingly, the behavior was rescued and returned to control levels of percent freezing. This suggests that CORT contributes to normal formation of fear memories. However, this work did not investigate the effect chronic stress has on CORT and in turn fear memory formation.

Conrad *et al*. repeated Pugh *et al*.'s work by utilizing metyrapone, a drug that prevents deoxycorticosterone to synthesize into CORT in the adrenal gland (Conrad *et al*., 2001; Sigalas *et al*., 2012; Donckier *et al*., 1986). This is different than Pugh *et al*.,'s work since it removes the confounding variables of surgery and the lack of adrenal glands. Moreover, Conrad *et al*. found similar results and found that blocking specifically CORT production was sufficient to dampen fear memory (2001). Following Conrad *et al*.'s work, McReynold *et al*. investigated whether CORT was sufficient to enhance memory consolidation (2014). An experiment to demonstrate this effect was to administer CORT systemically following inhibitory avoidance training, another fear conditioning memory test. Briefly, inhibitory avoidance training occurs when a rat is placed in a chamber with two sides: a light side (less preferable for a rat) and a dark side (more preferable for a rat). The rat starts in the light side and will move to the dark side. Once in the dark side, the rat will be given an aversive stimulus (foot shock). Following 24 hours, the rat is placed back in the same chamber and the researchers measure how long it takes before the rat moves to the dark side. The longer the latency, the greater the fear memory. This method is similar to contextual fear conditioning, but inhibitory avoidance training utilizes more of the prefrontal cortex to prevent the rat from entering the dark side, which does not occur in

contextual fear conditioning. From this test, McReynold found administering systemic CORT significantly enhances memory consolidation (McReynold *et al*, 2014).

McReynold *et al*.'s (2014) work focused on systemic CORT, but still needed to understand where CORT was acting in the brain. For this reason, they began to investigate the role of the BLA, CORT, and norepinephrine signaling. Specifically, they wanted to determine whether CORT was responsive via blocking beta-adrenergic receptors (β-ARs) in the BLA. To accomplish this, they used four groups: 1) Vehicle + Vehicle; 2) Vehicle + Propranolol (β-AR antagonist); 3) CORT + Vehicle; 4) CORT + Propranolol. Propranolol was administered at the BLA and CORT was administered systemically. The drugs were added post inhibitory avoidance training. Again, McReynold *et al*. found that CORT enhances fear memory. However, they also found that β-ARs in the BLA were important for CORT to enhance fear memory (McReynold *et al*., 2014).

McGaugh and Roozendaal's work supports McReynold *et al*.'s findings. McGaugh and Roozendaal's laboratories were the most in-depth when trying to understand the mechanism of CORT, β-AR signaling, and fear memory in the BLA. They investigated the mechanism of glucocorticoid and β-adrenergic signaling in enhanced fear memory via an acute stressor. More specifically, Roozendaal *et al*. first worked with the stress hormones utilizing the inhibitory avoidance test (2002). They added a corticotropin-releaseing hormone (CRH) agonist in the BLA of non-stressed rats and as expected, there was an enhancement in memory. Roozendaal and McGaugh continued their work by investigating the interaction between glucocorticoids and β-adrenergic receptors to modulate memory consolidation (Roozendaal, Quirarte, and McGaugh, 2002). They continued to utilize the inhibitory avoidance test and the BLA; however, they added β-AR antagonists and agonists in combination with glucocorticoid receptor (GR) antagonists. In

turn they unraveled the following mechanism of enhanced memory modulation: NE enhances memory consolidation via β-AR and α1 signaling; glucocorticoids enhance memory consolidation via α1 coupling; and α2 attenuates memory consolidation (Roozendaal, Quirarte, and McGaugh, 2002).

The stress response is regulated via the HPA axis; however, the regulation is sexually dimorphic. As described above, women often have low HPA responses compared to men, but in rodents, females have a stronger stress response following an acute stressor. In turn, females will have greater levels of CORT and adrenocorticotropic hormone (ACTH) when having a stressful event (Heinsbroek *et al*., 1991; Haleem *et al*., 1988; Kant *et al*., 1983). Normally, when an organism is challenged with a stressor, CORT is upregulated, it liberates energy throughout the body and in turn helps overcome the stressors. However, if CORT is dysregulated, the organism is more prone to stress disorders including anxiety (Faravelli *et al*., 2012).

The Role of Interleukin-1β on Fear Memory

Catecholamines and β-AR signaling facilitate fear memory; however, the downstream mechanism is unclear (LaLumiere *et al*., 2003; Camp and Johnson, 2015). One candidate molecule in this pathway is interleukin-1β (IL-1β), a proinflammatory cytokine. Specifically, catecholamines upregulate IL-1β via β-ARs (Johnson *et al*., 2005; Porterfield et al., 2012; Roozendaal, Quirarte, and McGaugh, 2002). Interestingly, chronic stress sensitizes β-AR signaling (Porterfield *et al*., 2012). Moreover, IL-1β can modulate fear memory (Avital *et al*., 2003; Goshen *et al*., 2007; Jones *et al*., 2015; Song, Phillips, and Leonard, 2003). Thus, it is a candidate molecule for the stress-induced enhancement of fear memories.

Avital *et al*. was one of the first labs investigating IL-1's role in fear memory. Specifically, Avital *et al* investigated whether IL-1 is necessary in mice for memory function. To accomplish this, mice lacking receptor IL-1 (IL-1rKO) were put through a contextual fear memory paradigm. The IL-1rKO mice had significantly less contextual fear memory compared to the controls (Avital *et al*., 2003). This study demonstrated that IL-1 contributes to contextual fear memory.

Following, Goshen *et al*. continued the work on IL-1, specifically IL-1β and contextual fear memory (2007). Goshen *et al*. tested whether IL-1β is necessary and/or sufficient for contextual fear memory. To accomplish this, Goshen *et al*. added IL-1β and IL-1RA (IL-1 antagonist) via intracerebroventricular administration. When given IL-1β, there was an inverted U-shaped dose curve. Moreover, low levels of IL-1β (1ng) was sufficient to enhance contextual fear memory. However, when given high doses of IL-1β, the mice had diminished contextual fear memory. When Goshen *et al*. removed IL-1 signaling via IL-1RA, they discovered that contextual fear memory was also diminished (2007). This suggests that IL-1 is important for normal contextual fear memory, and low levels of IL-1 can facilitate memory formation while high levels impair memory formation. Interestingly, Goshen et al., only found that IL-1RA diminishes contextual fear memory (hippocampus dependent) but not auditory-cued fear conditioning (hippocampus independent). This suggests that the hippocampus is a key brain region to study; however, Porterfield et al. work suggested the amygdala was more important over the hippocampus when chronic stress is an added variable (2012).

Porterfield *et al*. found that IL-1β signaling is sensitized in the amygdala under stressful conditions but not the hippocampus (2012). Specifically, Porterfield *et al*. administered isoproterenol, a β-AR agonist, to non-stressed and chronically stressed rats and measured IL-1

signaling throughout the brain. Porterfield et al. found isoproterenol to induce IL-1ß in both the amygdala and hippocampus; however, if the rats were chronically stressed prior to being given isoproterenol, the rats had a significantly greater increase in IL-1 production in the amygdala, but not the hippocampus (2012). Porterfield *et al*. did not disassociate the subregions of the amygdala, so it is unknown whether the increase in IL-1 was within the BLA specifically (2012). The data suggest that under chronic stress, IL-1 signaling can become sensitized within the amygdala that could mediate an enhancement in fear memory formation.

Chronic Stress

Currently, the literature is limited to a few studies in regard to chronic stress and memory tests. When female rats were chronically stressed via 6-hour restraints for 21 days, they performed better on the radial arm maze, a test to measure spatial memory (Bowman *et al*., 2001). On the other hand, males decreased in performance following chronic stress (Luine *et al*., 1994). Another test utilized was the object recognition task, which measures whether a rat can remember novel objects. Following 6-hour restraints for 7 days, rats completed the object recognition task. Interestingly, females had significantly better memory following chronic stress, whereas males had dampened memory (Beck and Luine, 2002). The data suggest overall that females have enhanced memory and learning following chronic stress; however, the contextual fear memory test has been limited following chronic stress.

There is overwhelming evidence that indicates chronic stress plays a vital role in both stress hormone sensitization and fear memory. Our laboratory previously investigated whether chronic stress sensitizes contextual fear memory in males (Camp and Johnson, 2015). Camp and Johnson chronically stressed adult male rats via a four day chronic stress paradigm. On the fifth

day, the rats were placed in conditioning chambers and administered two foot shocks to stimulate a fearful memory. The rats were then returned to their home cages and 24 hours later the rats were placed back in the same conditioning chamber and freezing behavior was recorded. Animals with prior stress exposure showed exaggerated freezing behavior when placed back in the conditioning chamber, suggesting that chronic stress sensitizes contextual fear memory compared to control. Moreover, Camp and Johnson investigated the mechanism of sensitization by administering either propranolol, a beta-adrenergic receptor antagonist (β-AR) or saline to a subgroup of animals prior to fear conditioning. The data revealed a significant interaction between drug treatment and stress exposure; propranolol treatment had no effect in non-stressed control animals but blocked the exaggerating freezing behavior in animals with prior stress exposure (Camp and Johnson, 2015). It suggests that β-ARs are important for chronic stress induced enhanced contextual fear memory. Currently, it is not clear what the downstream mechanism by which β-ARs facilitate fear memories in chronic stress animals. In addition, all the previous work was completed in male rats, thus more work is needed to determine whether females are susceptible to stress-induced enhanced contextual fear memory.

Our laboratory previously investigated whether the stress response is sensitized following chronic stress (Lowrance *et al*., 2016). To accomplish this, Lowrance *et al*. used the same four day chronic stress protocol utilized in Camp and Johnson's work. Following the four days, rats were restrained, and serial blood samples were collected. The data revealed that chronically stressed rats had an exacerbated CORT production compared to control rats (Lowrance *et al*., 2016). This suggests that the HPA axis is sensitized following chronic stress and this sensitization of the CORT response could be a potential method for the enhanced contextual fear memory (depicted in Figure 2) (Johnson *et al*., 2002; Bhatnagar *et al*., 1998; Akana *et al*., 1992).

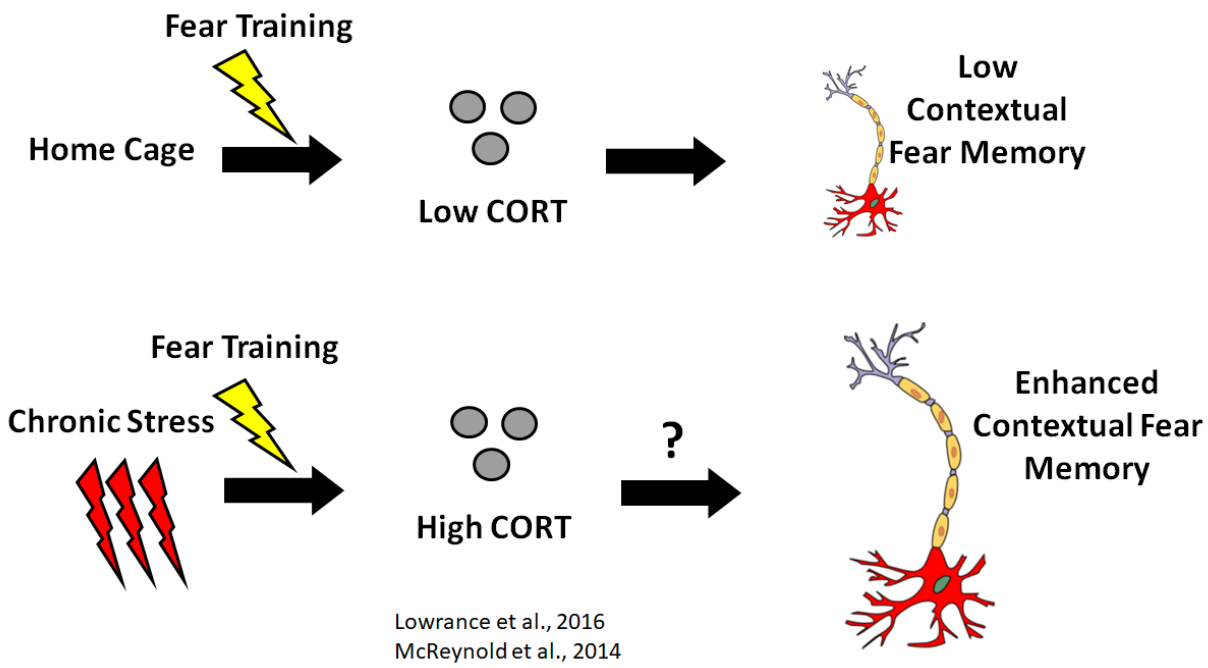

Figure 2. CORT Hypothesis Overview: When rats are not stressed, they have low CORT. In turn, there is low contextual memory. However, when rats are chronically stressed, CORT becomes sensitized (Lowrance *et al*., 2016). Since CORT enhances fear memory (McReynold *et al*., 2014), our hypothesis proposed that the elevation of CORT from chronic stress is responsible for the enhancement of contextual fear memory.

Conrad *et al*. investigated the effect of chronic stress on fear memory (2001). However, this stress paradigm was different than Camp and Johnson (2015) and Lowrance *et al*. work (2016). Specifically, this stress paradigm is 21 days of restraint for 6 hours each day. Interestingly, Conrad *et al*. did not find chronic stress to enhance fear memory. Instead, they found that stress has an interaction with metyrapone, which prevents the synthesis of CORT (2001). They showed that chronically stressed rats given metyrapone have less fear memory compared to non-stressed rats given metyrapone. Conrad *et al*.'s work is contradicted by later studies from Conrad's laboratory where investigations (Hoffman *et al*., 2014; Hoffman *et al*., 2015) actually found that chronic stress does enhance fear memory.

In addition to CORT, norepinephrine (NE) is upregulated following stress. The literature suggests NE signaling enhances fear memory formation; however, when NE signaling is blocked via β-AR antagonists in normal (non-stressed) animals, there was no effect (Inoue *et al*., 2006). Interestingly, if rats were chronically stressed prior, there was a significant decrease compared to chronically stressed rats administered with saline (Camp and Johnson, 2015). This suggests priming the stress response alters the mechanism of fear memory. Currently, it is unclear how stress alters NE's downstream signaling in relation to enhanced fear memory. This work utilized the novel idea that the sensitization of IL-1β following chronic stress is at levels that facilitate memory formation (Figure 3).

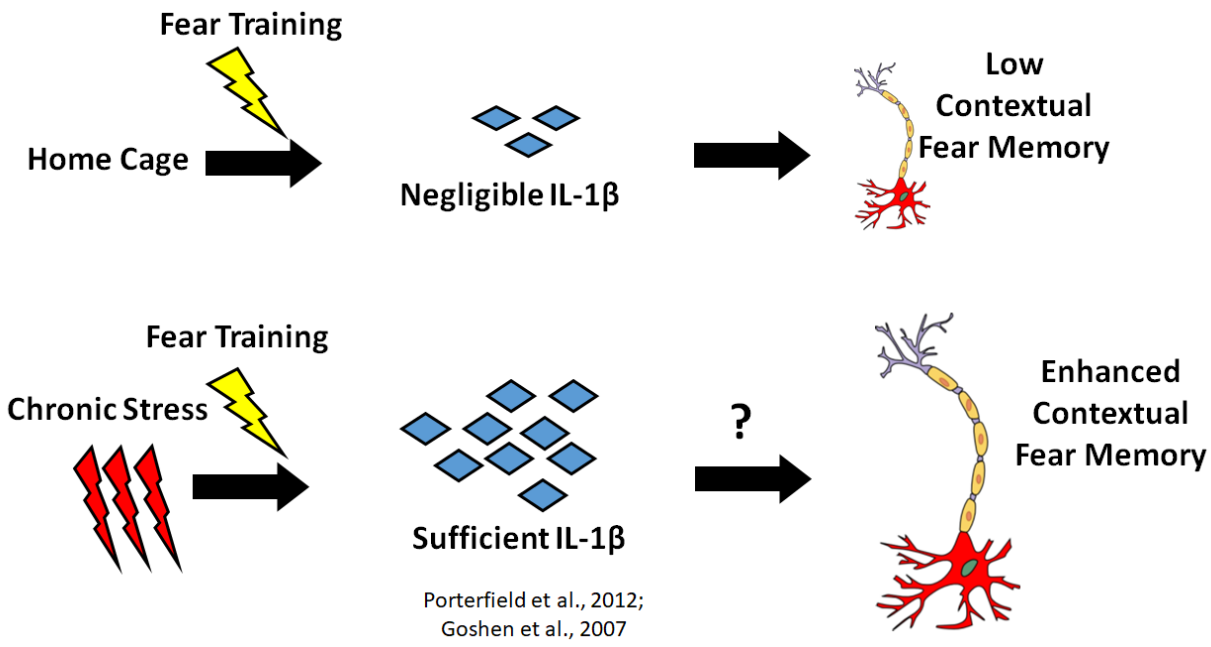

Figure 3. IL-1β Hypothesis Overview: When rats are not stressed, they have low IL-1β. In turn, there is low contextual memory. However, when rats are chronically stressed, IL-1β becomes sensitized (Porterfield *et al*., 2012). Since IL-1β enhances contextual fear memory (Goshen *et al*., 2007), our hypothesis proposed that the elevation of IL-1β from chronic stress is responsible for the enhancement of contextual fear memory.

Research Objectives

Aim 1) Do female rats develop enhanced fear memory following chronic stress?

Clinically, women have greater prevalence of anxiety, specifically PTSD behavior (Kessler *et al*., 2005); however, the rodent literature is lacking evidence of whether females are susceptible to enhanced fear memory following chronic stress. To investigate this question, we used two treatment groups: 1) Chronically stressed female rats; 2) Non-chronically stressed female rats. The chronically stressed female rats were put through the chronic stress paradigm explained in Table 1, while the non-chronically stressed rats were in their home cage throughout the protocol. The two treatment groups went through the fear conditioning paradigm (Figure 4). The fear conditioning paradigm works as follows: Individual rats are placed inside a novel conditioning chamber for a total of 5 min. At min 2 and min 4, rats received a 2 sec foot shock (1.5mA). The rat is removed and placed back in its home cage for 24 hrs. The rat is then placed back in the same conditioning chamber and percent freezing is measured. Throughout the entire fear conditioning process, rats are being video recorded. Videos were reviewed via a researcher blinded to the treatment groups. This contextual fear memory model is hippocampus dependent and also utilizes the BLA to form the associative memory between stressor and context (Maren, 1999; Gale et al., 2004; Phillips and LeDoux, 1992; Curzon, Rustay, and Browman, 2011). We predicted that chronically stressed female rats would have enhanced fear memory compared to non-chronically stressed female rats. The data were analyzed using a two-tailed t-test (alpha= 0.05). The sample size was n=8 group since our laboratory found significant effect using this protocol in males (Camp and Johnson, 2015).

Table 1. Chronic Stress Paradigm:

Day	AM	PM
1	Restraint	Food Deprivation
2	Fox Odor	Constant Light
3	Restraint	Wet Bedding
4	Forced Swim Test	-
5	Fear conditioning	-
6	Memory Measurement	-

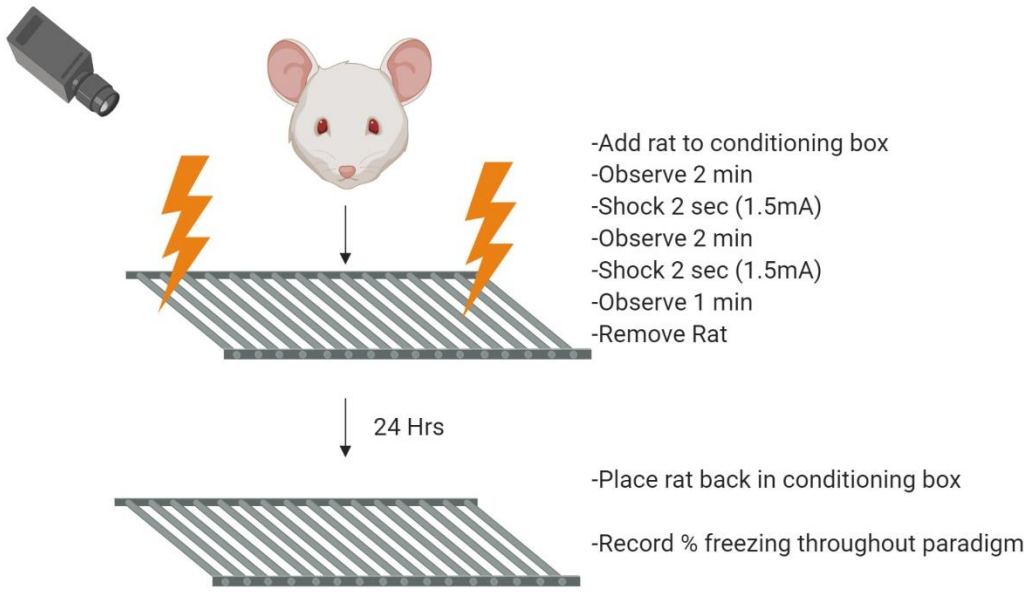

Figure 4. Chronic Stress Paradigm: Individual rats are placed inside a novel conditioning chamber for a total of 5 min. At min 2 and min 4, rats received a 2 sec foot shock (1.5mA). The rat is removed and placed back in its home cage for 24 hrs. The rat is then placed back in the same conditioning chamber and percent freezing is measured. Throughout the entire fear conditioning process, rats are being video recorded.

Aim 2) What is the role of CORT in chronic stress enhanced fear memory?

2.1. Is the CORT response sensitized to fear conditioning following chronic stress?

We previously demonstrated that rats that are chronically stressed have a primed CORT response to subsequent stress exposure (Lowrance *et al*., 2016). Fear conditioning causes an array of endocrine changes. CORT is of particular interest because there is strong evidence that CORT is sufficient to enhance fear memory in rats (McReynolds *et al*., 2014). In this study, we investigated whether CORT is elevated following fear conditioning and whether chronic stress augments the CORT response. We utilized two treatment groups: Rats under chronic stress (Table 1) and Non-stressed rats (n=3-5 per group). Following chronic stress (or not chronic stress) rats underwent fear conditioning (Figure 4). Twenty minutes following fear conditioning, we euthanized and collected blood since this is when CORT was sensitized in the Lowrance *et al*. study (2016). We predicted that CORT would be greater in chronic stress rats, which was analyzed via a two-tailed t-test (alpha=0.05).

2.2. Does blocking CORT peripherally during conditioning prevent chronic stress enhancement in fear?

This work has been previously demonstrated in Conrad *et al*.'s work where they used a 6 hour restraint for 21 days; however, their data suggest that there is no enhancement in memory following chronic stress (Conrad *et al*., 2001). This could be a result of the intensity of their stress protocol. This is unexpected since our laboratory and a future paper published from Conrad *et al*. laboratory demonstrated that chronic stress does enhance fear memory (Hoffman *et al*., 2015; Camp and Johnson, 2015). We needed to confirm this phenomenon before continuing with aim 2. We used the following treatment groups: 1) Stressed rats given metyrapone; 2)

Stressed rats given saline; 3) Non-stressed rats given metyrapone; 4) Non-stressed rats given saline. Stressed rats went through the stress paradigm explained in Table 1. Metyrapone (100mg/kg) or saline were administered peripherally 2 hours prior to fear conditioning (Figure 4). We predicted metyrapone would dampen fear memory in both stressed and non-stressed rats. However, we also predicted that chronically stressed rats given saline would have the greatest amount of percent freezing compared to every other condition. Sample size was n=12-16 /group since it was previously demonstrated to have a significant effect in a similar study (Conrad *et al*., 2001). The data was analyzed via two-way ANOVA test (alpha=0.05).

2.3. If CORT is necessary for the enhancement of fear memory, does blocking CORT in the BLA prevent chronic stress enhancement in fear.

We currently know the literature suggests that chronic stress sensitizes both the CORT response and fear memory (Lowrance *et al*., 2016; Camp and Johnson, 2015). However, it was unknown whether CORT was responsible for the chronic stress enhancement in fear memory. To answer this question, we will have the following treatment groups: 1) Stressed rats with RU38486, a glucocorticoid receptor (GR) antagonist (3ng in 0.2 uL); 2) Stressed rats with saline; 3) Non-stressed rats with RU38486; 4) Non-stressed rats with saline. Stressed rats went through a four day chronic stress paradigm (Table 1). RU38486 was used over a mineralocorticoid receptor antagonist because Roozendaal *et al*. found that GRs facilitate memory consolidation (2009). RU38486 will be administered in the BLA 30 min prior to fear conditioning (Figure 4). We predicted RU38486 would have a small dampening effect on non-stressed rats. Moreover, we expected RU38486 to dampen the stressed rats' memory equivalent to the non-stressed rats. We also expected chronic stress to enhance memory following fear conditioning. These data

were analyzed via a two-way ANOVA (alpha=0.05). Sample size was n=7-12 /group since it was previously demonstrated to have a significant effect in a similar study (Conrad *et al*., 2001).

Aim 3) What is the role of IL-1β in chronic stress enhanced fear memory?

3.1. What is the effect of IL-1β in the BLA on enhanced fear memory in non-stressed rats?

Goshen *et al*. found that IL-1β has a dose dependent response (inverted U-shaped) for enhanced contextual fear memory when injected via intracerebroventricular (ICV) administration (Goshen *et al*., 2007); however, the location where IL-1β acts to alter memory was still unknown. Given the role of the BLA in associative learning, we tested if IL-1β is sufficient to enhance memory consolidation. Rats were cannulated at the BLA. Following surgery, the rats were given three weeks to recover. All rats went through fear conditioning (Figure 4). Following, we administered 0.5 μL of IL-1β in a dose dependent manner in the BLA: 1) 0.0 ng/ul; 2) 0.01 ng/ul; 3) 0.1 ng/ul; 4) 1.0 ng/uL immediately following fear conditioning. After 24 hours, the rats were placed in the same conditioning chamber and measured their memory of the context (measure freezing behavior). We expected to see low levels of IL-1β (0.01 ng/uL and 0.1 ng/ul) leading to enhanced memory, but high levels (1.0 ng/ul) leading to dampened memory. This was analyzed via a one-way ANOVA (alpha=0.05). Sample size were n=10-14/group since it was previously demonstrated to have a significant effect in a similar study (Goshen *et al*., 2007).

3.2: What are the effects of chronic stress on IL-1β and IL-1RA signaling in the BLA when given fear conditioning?

The literature was unclear on the exact role that IL-1β has on memory consolidation and chronic stress. Our laboratory previously demonstrated the amygdala has an upregulation of IL-1β protein following chronic stress (Porterfield *et al*., 2012). However, to the best of our knowledge, IL-1β signaling has never been measured in the BLA following chronic stress, which would provide vital insight on the potential mechanism of IL-1β and memory. We have preliminary data to suggest that non-chronically stressed rats trend to have enhanced IL-1RA mRNA 60 minutes after an acute stressor (foot shock). For this reason, we tested the hypothesis that chronic stress sensitizes IL-1β and IL-1RA mRNA in the BLA following the acute stressor. To answer this question, we had four treatment groups, 1) Chronic stress + Fear Conditioning; 2) Chronic Stress + No Fear Conditioning; 3) Non-stressed + Fear Conditioning; 4) Non-Stressed rats + No Fear Conditioning (n=6-10/ group). Stressed rats were put through a four day chronic stress paradigm as described in Table 1. The BLA was then micropunched and analyzed through real-time PCR to measure gene expression of IL-1β, IL-1RA, and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH- control). We expected both IL-1β and IL-1RA (antagonist) to be upregulated in chronically stressed rats compared to non-stressed, which was analyzed via a two-way ANOVA (alpha=0.05).

3.3. Is IL-1β necessary in the BLA for the exaggerated fear memory produced from chronic stress?

Chronic stress has previously been demonstrated to enhance IL-1β signaling (Porterfield *et al*., 2012). In addition, IL-1-β has a dose dependent effect on memory (Goshen *et al*., 2007); however, IL-1β has never been investigated as mediating the enhanced memory produced following chronic stress. To test this idea, we had four treatment groups: 1) Chronic Stress + IL-

1RA; 2) Chronic Stress + Saline; 3) No Stress + IL-1RA and 4) No Stress + Saline. Stressed rats were put through a four day chronic stress paradigm (Table 1). Rats were given 0.5ul/side of 25ng/uL IL-1RA in the BLA immediately following fear conditioning. After 24 hours, the rats were placed in the same conditioning chamber to measure their memory of the context. This approach is the current best method to measure contextual fear memory since the BLA has been identified as a key brain region in this task. Specifically, without the BLA, rats are unable to remember the dangerous context following the initial fear conditioning (Maren, 1999; Gale et al., 2004; Phillips and LeDoux, 1992). We expected IL-1RA to have a small dampening effect on control rats. We also expected stressed rats to have enhanced fear memory compared to controls. However, we expected stressed rats given IL-1RA (group 1) to be protected from the chronic stress induced enhanced memory phenomenon. We expected stressed rats given IL-1RA (group 1) to have similar memory to non-stressed rats given saline (group 4). This was analyzed via a two-way ANOVA (alpha=0.05). Sample size will be n=7-12/group since it was previously demonstrated to have a significant effect in a similar study (Jones *et al*., 2015).

Chapter 2

The Role of CORT on Fear Memory Article

Abstract:

Following a stressful event, the hypothalamus - pituitary - adrenal (HPA) axis mediates the release of the stress hormone cortisol (corticosterone in rodents; CORT). Elevated CORT binds to glucocorticoid receptors to mediate physiological responses including facilitating memory formation. Previous work from our laboratory demonstrated that male rats exposed to chronic stress demonstrate enhanced contextual fear memories and enhanced CORT responses to subsequent stress exposure. The experiments here tested whether chronic stress enhances fear memory formation in female rats and whether the sensitized CORT response in chronic stress rats contributes to their enhanced freezing behavior (test of fear memory). Studies first examined CORT responses to contextual fear conditioning in male and female rats and examined whether chronic stress enhanced the formation of contextual fear memories 24h later. Studies then used metyrapone, a CORT synthesis inhibitor, to investigate whether blockade of plasma CORT would eliminate the chronic stress-induced enhancement in contextual fear memory. Finally, the

glucocorticoid receptor antagonist, RU38486, was administered into the BLA 30 minutes prior to fear conditioning to determine if it would block the enhancement in fear memory formation in chronic stress animals. Results show that female rats have greater CORT responses than males and chronic stress sensitizes the CORT response to fear conditioning in both sexes. However, female rats do not show enhanced contextual fear memory following chronic stress. Chronically stressed male rats show greater freezing behavior during fear conditioning (acquisition phase) and show greater freezing behavior 24h later when returned to the context previously paired with foot shocks (fear memory). Metyrapone dampens contextual fear memory in all animals but does not eliminate the enhancement in freezing behavior in chronic stress animals. Interestingly, cannulated rats exposed to chronic stress fail to show sensitized acquisition of freezing behavior as seen in non-cannulated rats. RU38486 had no effect on fear memory formation when administered directly into the BLA. Collectively, these studies indicate sensitized CORT responses in chronically stressed animals is likely not the mechanism by which chronic stress facilitates memory formation.

Introduction:

Chronic stress impacts an individual's behavior such as restlessness and avoidance of non-harmful stimuli, which are more broadly classified as anxiety (Eiland and McEwen, 2012). Clinically, diagnoses of anxiety disorders range from generalized anxiety disorder, panic disorder, social anxiety disorder, and post-traumatic stress disorder (PTSD). These diagnoses are all characterized by an enhanced fear response and more specifically an underlying enhanced fear memory. Notably, women are more likely than men to develop anxiety (Kessler *et al*., 2005); however, research conducted on anxiety in rodents is mostly completed in males. Thus,

an investigation into whether female rodents are susceptible to enhanced fear memory formation following chronic stress is needed.

The brain region currently thought to be responsible for the formation of enhanced fear memories is the basolateral amygdala (BLA). Maren *et al*. previously demonstrated that lesioning of the BLA impedes the association of fear memories, which was shown in rats that underwent fear conditioning following lesioning of the BLA and were unable to learn that an area was dangerous following the initial fear conditioning (Maren, 1999). Additionally, the BLA is important for stress hormones to modulate memory (Paré, 2003). Roozendaal and McGaugh have demonstrated that the BLA is a key brain region for memory consolidation and the site of action for stress hormones to facilitate fear memory formation following a stressful event (Roozendaal *et al*., 2002; Roozendaal, Quirarte, and McGaugh, 2002; Roozendaal et al., 2009).

Corticosterone (CORT), a stress hormone important for liberating energy in response to a stressor has been demonstrated to have a role in modulating memory formation. McReynolds *et al*. investigated whether CORT was sufficient to enhance memory consolidation by administering a single injection of CORT immediately after inhibitory avoidance conditioning. They demonstrated that CORT was sufficient to enhance memory consolidation (McReynolds *et al*., 2014). Additionally, Roozendaal *et al*. have worked to determine the molecular mechanisms of fear memory formation in rodents by demonstrating that glucocorticoids enhance memory consolidation via α1-adrenergic receptor coupling to glucocorticoid receptor (GR); however, their laboratory only utilized acute stressors (Roozendaal, Quirarte, and McGaugh, 2002). Chronic stress often primes the CORT response to subsequent acute stress exposure (Lowrance *et al*., 2016). Chronic stress has also been demonstrated to enhance fear memory formation in rodents (Camp and Johnson, 2015). It is currently unknown whether the chronic stress

enhancement of fear memory is caused by the chronic stress sensitization of the CORT response. The present work investigated the role of CORT in the chronic stress enhancement of fear memory formation in both male and female rodents. It was hypothesized that CORT is necessary for the chronic stress enhancement of fear memory.

Studies first characterized whether CORT production is enhanced in chronically stressed male and female rodents following fear conditioning. Second, we tested whether female rats develop enhanced fear memories. Third, we tested whether CORT is necessary systemically for chronic stress enhanced fear memories by blocking CORT production. Finally, we administered the GR antagonist, RU38486, directly into the BLA to determine if CORT's binding to GRs was necessary for the formation of chronic stress enhanced fear memories.

Methods:

Animals and Housing: Female and male Fischer rats were used (Harlan, Indianapolis, Indiana) since this strain is highly stress responsive and more susceptible to stress-induced pathology compared to many other rat strains (Camp *et al*., 2012; Porterfield *et al*., 2011; Uchida *et al*., 2008). Adult rats (250–350 g) were single-housed in standard rat cages, and given access to food and water *ad libitum*, except when undergoing food restriction stress (see below). Rats were kept on a 12:12 h light–dark cycle (lights on at 08:00). All animals were handled according to the Animal Welfare Act and The Guide for the Care and Use of Laboratory Animals. The Kent State University Institutional Animal and Care Committee approved all procedures.

Chronic Stress Paradigm: Male Fischer rats were exposed to a series of stressors following an established protocol (Camp *et al*., 2012) or were left undisturbed as home cage control animals. This four day repeated stress paradigm was chosen since we previously demonstrated that it

results in increased NE turnover in the amygdala, sensitized β-AR mediated responses, and enhanced fear conditioning to contextual cues (Porterfield *et al*., 2012; Camp *et al*., 2012; Camp and Johnson, 2015). On the morning (08:00–10:00) of day 1, chronic stress rats were placed in DecapiCone rodent restrainers (Braintree Scientific, Inc., Braintree, MA) for 60 min, before being returned to their home cage. At 15:00 h, food was removed from chronic stress rat cages for 18 h. On day 2, chronic stress rats were placed in novel habitats, containing 35 μL trimethylthiazoline (a component of fox feces) to simulate predator odor. Rats were then placed back in their home cages but were housed in constant light conditions overnight. On the morning of day 3, animals were exposed to restraint stress again for 60 min, then at 15:00 h their bedding material was dampened with approximately 1500 mL distilled water. On day 4, subjects were exposed to forced swim for 5 min in glass cylinders measuring 49 × 18.7 cm (inner height and diameter, respectively) filled approximately to the 37.5 cm line with water at a temperature of 21°C. Following this task, subjects were placed in cages containing dry bedding. Following chronic stress and prior to behavioral and physiological observations, the subjects' weights were recorded. Control rats were in their home cage for the entire duration of the chronic stress paradigm.

Fear Conditioning Paradigm: One day following the stress protocol (day 5), rats were placed in a 21.59 × 21.59 × 27.94 cm conditioning chamber (Lafayette Instrument Company, Lafayette, IN) with a floor consisting of a series of electrically conductive steel bars for a total of 5 min. At min 2 and min 4, rats received a foot shock (1.5 mA for 2 s). Following fear conditioning, rats were placed back into their home cages. Rats were recorded using a C615 HD Webcam (Logitech, Silicon Valley, CA) to measure acquisition during the time of fear conditioning.

Acquisition time points were separated into “0-2 min”; “2-4 min”; and “4-5 min” for analysis of freezing behavior.

Assessment of Fear Memory: Twenty-four hours later, subjects were placed back into the conditioning chamber and behavior was recorded for 15 min and freezing behavior was evaluated. Freezing behavior was defined as complete immobility, except for movements necessary for respiration. Scoring was performed by a trained researcher blind to group assignment. Scores were obtained by checking the video every 10 sec for 15 min, and one point was assigned for each instance of freezing behavior, with a maximum possible score of 90 pts.

Tissue Collection*:* Rats were submitted to the fear conditioning paradigm on day 5 as described above. For study 1, rats were decapitated immediately following fear conditioning to collect blood and measure plasma CORT. The CORT was measured using the Corticosterone ELISA Kit (Enzo, NY; LOT: 04281702; CAT: ADI-901-097) and followed the respective protocol. In study 3 and study 4, brains were extracted and frozen in a solution of isopentane and dry ice held at -20°C for approximately 60 sec. The brains were later utilized to record whether the cannula was located at the BLA.

Cannula Implantation: Adult rats were anesthetized with 2% isoflurane and placed in a stereotaxic apparatus. A burr hole was drilled posterior to bregma using the following coordinates: [AP:−2.9mm; ML: +/- 4.5 mm lateral to the midline; DV: 8.2 mm below skull surface]. Stainless-steel guide cannula (Plastics-One Inc., VA) were secured to the skull with three stainless-steel screws, super glue, and dental cement, and was closed by a dummy cannula (Plastics-One Inc.). Animals were administered Ketoprofen (2.0 mg/kg in 25% DMSO and 0.9% saline) immediately prior to surgery and again 24hr after surgery. Experiments were performed

following 3-4 weeks of recovery. The location of the cannula was verified at the end of the study by extraction of the brain and locating the cannulation blood trail. If a cannula was found to be misplaced, the rat was excluded from the study.

Metyrapone Injections:

Study 3: Two hrs prior to fear conditioning, 100 mg/kg of metyrapone (LOT:H; CAT:1443001) or saline was administered systemically. Rats were then returned to their home cage for the 2 hr duration.

Study 4: 30 min prior to conditioning, 0.2 µL of saline + 2% EtOH or RU38486 (LOT:WXBC6749V; CAT:M8046; a glucocorticoid receptor antagonist) + 2% EtOH (3ng with in 0.2 uL) was administered into the BLA using an internal 33GA fit guide (injector) cannula with a 0.5 mm projection (Plastics-One Inc., VA). RU38486 was used over a MR antagonist because Roozendaal *et al*. found that GRs facilitate memory consolidation (2009). The injector cannula was connected to polyethene tubing (Stoelting, Wood Dale, IL) and a Hamilton 5uL syringe (Stoelting,Wood Dale, IL). Solutions were administered at a constant rate for 30 sec, and the injection cannula was removed 30 sec following the termination of the injection to avoid spillage from the guide cannula. Rats were tested 24 h later for contextual fear memory, as described above.

Statistical Analysis:

Study 1: The effect of chronic stress on CORT between sexes: A two-way ANOVA was used to analyze statistical significance (alpha = 0.05).

Study 2: The effect of chronic stress on female contextual fear memory: A t-test was used to analyze statistical significance (alpha=0.05).

Study 3: The effect of metyrapone on contextual fear memory in chronic stressed rats: A repeated measures analysis was utilized to determine statistical significance in acquisition. A two-way ANOVA test was used to analyze statistical significance in contextual fear memory (alpha = 0.05).

Study 4: The effect of RU38486 in the BLA on contextual fear memory: A repeated measures analysis was utilized to determine statistical significance in acquisition. A two-way ANOVA test was used to analyze statistical significance in contextual fear memory (alpha = 0.05).

Results

Study 1: The effect of chronic stress on CORT between sexes

To understand the sensitization of CORT following chronic stress, baseline CORT was first measured in both male and female rats. Following, rats were separated into either chronic stress or no stress groups. Following the chronic stress protocol, all rats underwent fear conditioning and were immediately decapitated to determine the change in plasma CORT. A two-way ANOVA revealed a significant main effect of CORT in chronically stressed rats compared to non-stressed rats [$F(1,17) = 10.26$, $p = 0.005$]. In addition, the two-way ANOVA revealed a significant main effect of sex [$F(1,17) = 5.88$, $p = 0.027$] in which females had a significantly greater CORT response compared to males. There was no interaction present [$F(1,17) = 1.395$, $p=.254$] (Figure 5).

Study 2: The effect of chronic stress on female contextual fear memory

To determine whether female rats are susceptible to chronic stress enhancement in fear memory, female rats were separated into either chronic stress or non-stress groups and all rats went through contextual fear memory paradigm. There was no significant difference in freezing behavior between chronic stress and non-stressed groups [t (14) = -0.356, p = 0.727; Figure 6)

Study 3: The effect of metyrapone on contextual fear memory in chronic stressed rats

To examine the role of CORT on fear, metyrapone (CORT synthesis inhibitor) or saline was administered systemically in chronic stress or non-stressed male rats. A repeated measures analysis revealed that during the acquisition of the fear memory, chronically stressed rats had a significant increase in freezing [F (1,44) = 2.942, p=0.047, one-tailed] (Figure 7). In addition, there was a significant effect of time during the acquisition phase of fear conditioning [F (1,44) = 36.11, p = 0.0001] (Figure 7). However, the data did not reveal a significant effect of metyrapone during acquisition [F (1,44) = 1.438, p = 0.237)). Moreover, there was no interaction discovered [F(1,44) = 1.771, p = 0.190] (Figure 7).

A two-way ANOVA analysis revealed a significant main effect of chronic stress in which chronically stressed rats had a significantly greater contextual fear memory compared to non-stressed rats [F (1,44) = 4.13, p = 0.048]. The two-way ANOVA analysis also revealed a significant main effect of metyrapone administration in which metyrapone dampened fear memory [F (1,44) = 10.98, p = 0.002]. There was no interaction between metyrapone treatment and stress condition [F (1,44) = 0.183, p = 0.671] (Figure 8).

Study 4: The effect of RU38486 in the BLA on contextual fear memory

To determine whether the elevation in CORT production during fear conditioning influences the formation of fear memory, RU38486 was administered directly into the BLA 30 min prior to fear conditioning. The results of a repeated measures analysis revealed a significant effect of time between 0-2 min, 2-4 min, and 4-5 min [$F(1,30) = 173.72$, $p = 0.0001$] during acquisition of the fear memory (Figure 9). This suggests that the acquisition of the rats was intact. There was no main effect of RU38486 during acquisition [$F(1,30) = 0.062$, $p = 0.806$], but there was a significant main effect of chronic stress [$F(1,30) = 5.971$, $p = 0.021$]. A two-way ANOVA did not reveal an effect of chronic stress [$F(1,30) = 0.337$, $p = 0.566$] or RU38486 [$F(1,30) = 0.188$, $p = 0.668$] when measuring fear memory 24 hr later (Figure 10).

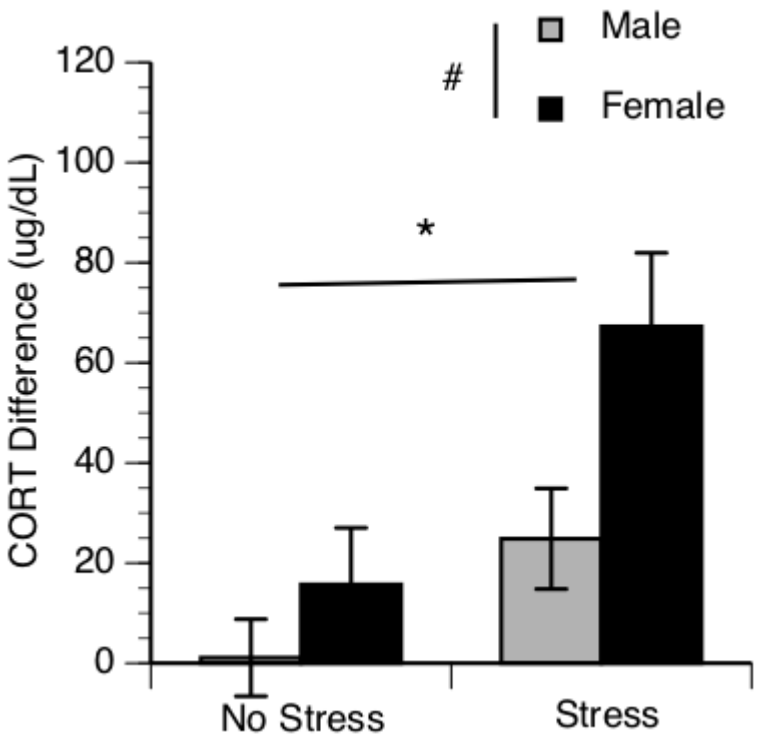

Figure 5. Chronic stress sensitizes the CORT response: Baseline CORT was collected of male (n=10) and female (n=11) rats. Rats were then either chronically stressed (n=11) or not chronically stressed (n=10). All rats then received fear conditioning. Immediately following fear conditioning, trunk blood was collected. The CORT difference (CORT Post Fear Conditioning - CORT Baseline) is reported. * Effect of Stress $p<0.05$; # Effect of Sex $p<0.05$.

Figure 6. Female rats do not show enhanced contextual fear memory: Female rats were either not chronically stressed (n=8) or chronically stressed (n=8). Following all rats underwent the fear conditioning paradigm. Following 24 hours, freezing behavior was recorded.

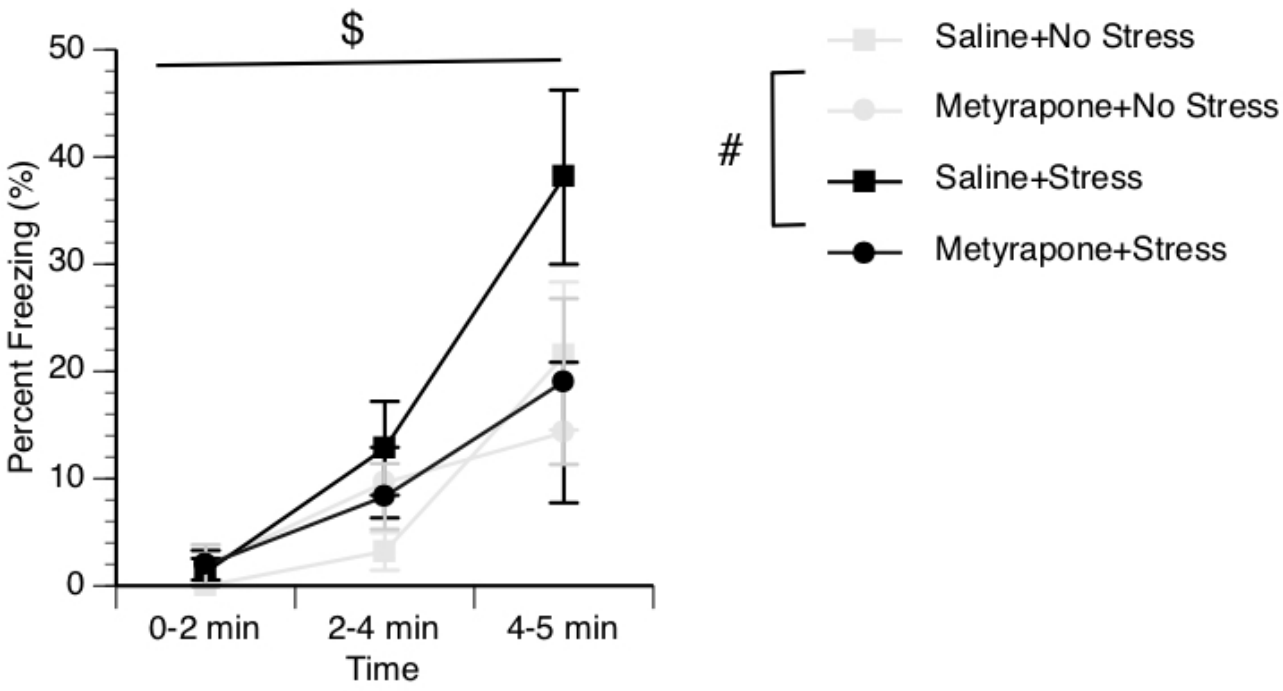

Figure 7. Rats that are stressed have sensitized acquisition to fear conditioning: Rats were either not chronically stressed (n=24) or chronically stressed (n=24). In addition, rats were administered saline (n=24) or metyrapone (n=24) 2 hr prior to fear conditioning. All rats underwent the fear conditioning paradigm (n=12/group). Throughout the fear conditioning paradigm (acquisition), rats were recorded for percent freezing. The freezing was separated 0-2 min (prior to foot shock); 2-4 min (after one foot shock); and 4-5 min (after two foot shocks). $ Effect of time $p<0.05$; # Effect of Stress $p<0.05$.

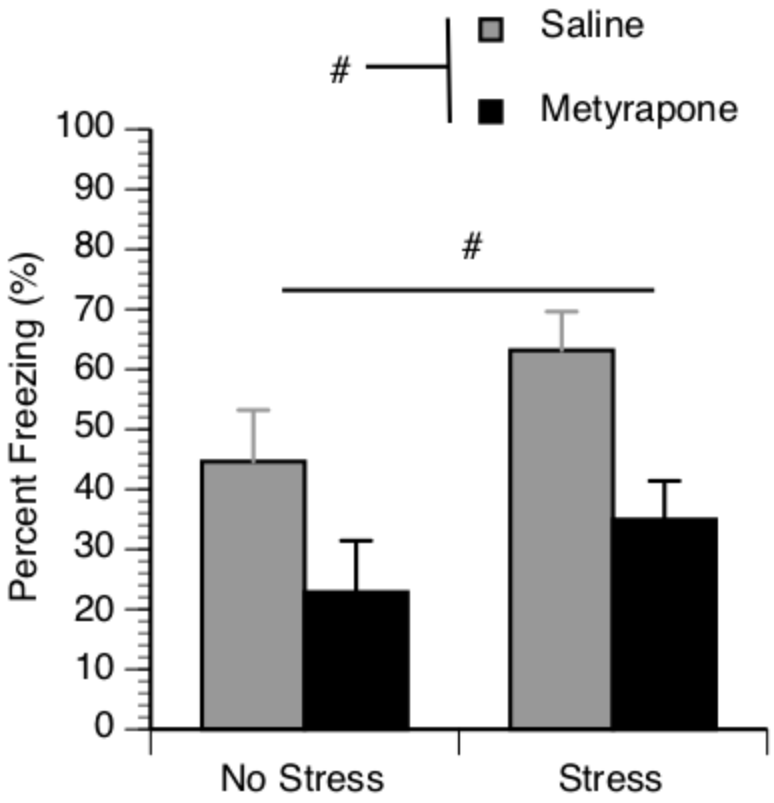

Figure 8. Chronic Stress sensitizes fear memory and metyrapone dampens fear memory: Rats were either not chronically stressed (n=24) or chronically stressed (n=24). Rats were administered saline (n=24) or metyrapone (n=24) via IP injection 2 hours prior to fear conditioning (n=12/group). All rats underwent the fear conditioning paradigm. Following 24 hours in home cage, rats were put in the same context and percent freezing was measured. The first five minutes of contextual fear memory is reported. # Main treatment effect p=<0.05.

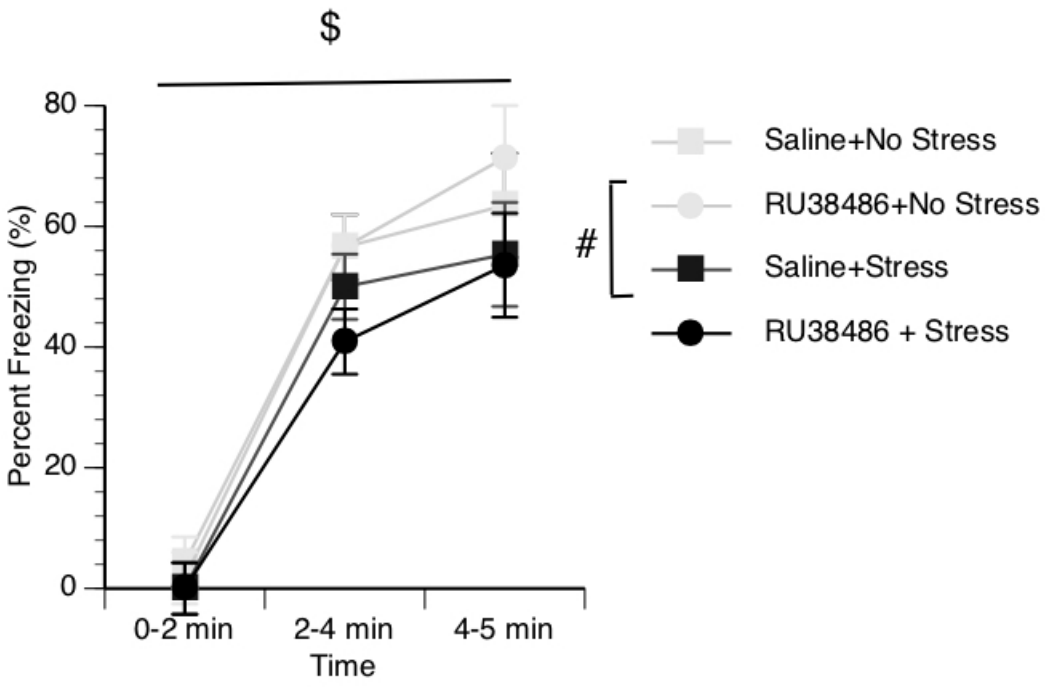

Figure 9. Cannulated chronically stressed rats fail to have enhanced fear acquisition: Rats were either not chronically stressed (n=18) or chronically stressed (n=16). Rats were either administered saline (n=17) or RU38486 (n=17) 30 min prior to fear conditioning. All rats underwent the fear conditioning (n=8-9/group). Throughout the fear conditioning paradigm (acquisition), rats were recorded for percent freezing. The freezing was separated 0-2 min (prior to foot shock); 2-4 min (after one foot shock); 4-5 min (after two foot shocks). $ Effect of time $p<0.05$; # Effect of Stress $p<0.05$.

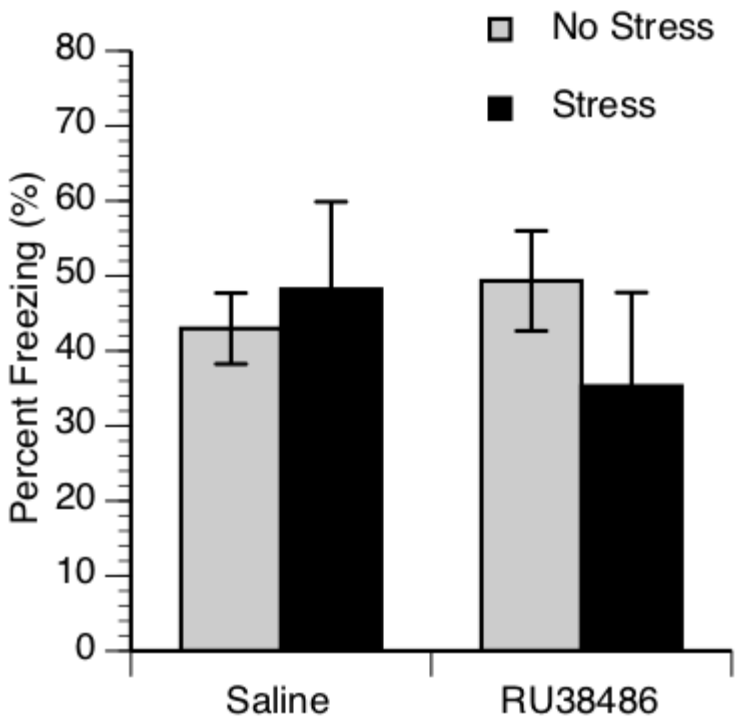

Figure 10. Chronically stressed rats failed to show enhanced fear memory: Rats were either not chronically stressed (n=18) or chronically stressed (n=16). Rats were either administered saline (n=17) or RU38486 (n=17) 30 min prior to fear conditioning. (n=8-9/group). All rats underwent the fear conditioning paradigm. Following 24 hrs, rats were placed in the same context and percent freezing was recorded.

Discussion:

Studies presented here investigated the role of CORT on contextual fear memory. It was discovered that chronic stress sensitizes the CORT response in both males and females with females having greater CORT responses compared to males. However, female rats do not show enhanced contextual fear memory following chronic stress. Male rats that undergo chronic stress have sensitized acquisition to fear conditioning and sensitized contextual fear memory 24 hrs after fear conditioning. Metyrapone has no effect on acquisition but dampens contextual fear memory 24 hrs later. Interestingly, chronically stressed rats that are cannulated had a significantly lower acquisition, but no change from RU38486. In addition, RU38486 failed to alter fear memory when administered directly into the BLA.

Chronic stress sensitizes contextual fear memory in males (Camp and Johnson, 2015; Jones *et al*., 2015); however, the molecular mechanism of this phenomenon was unclear. The literature suggested that CORT was a candidate molecule since chronically stressed rats have an enhanced production of CORT to a subsequent stressor (Lowrance *et al*., 2016), and CORT is sufficient to enhance memory consolidation (McReynold *et al*., 2014). For this reason, this work investigated CORT's role in fear memory, specifically in the BLA.

Camp and Johnson (2015) demonstrated that chronic stress enhances contextual fear memory in male rats. One of the limitations of their work is that females were not utilized, thus it was unknown whether female rats are susceptible to chronic stress enhanced contextual fear memory like males. This is surprising since Kessler *et al*. found that clinically, women are more likely to develop anxiety and twice as likely to develop PTSD (Kessler *et al*., 2005). For this reason, we first characterized the CORT response to fear conditioning in females compared to males. To the best of our knowledge, this is the first work that demonstrated CORT is sensitized

in males and females following chronic stress. Moreover, females had an even greater CORT response compared to males (Figure 5). This is significant because it would initially suggest that the sensitized CORT is a prime candidate for the mechanism behind chronic stress induced contextual fear memory. For this reason, we repeated Camp and Johnson (2015) chronic stress protocol but with female rats. Surprisingly, even when female rats demonstrate greater CORT responses to fear conditioning compared to males, females failed to develop chronic stress induced enhancement in contextual fear memory (Figure 6). This is the first indication that sensitized CORT responses following chronic stress is not sufficient to enhance fear memories. Since female rats were not susceptible to chronic stress-induced enhancement in fear memory, the remaining studies only utilized male rats.

Conrad *et al*. investigated the effects of chronic stress on fear memory. However, their chronic stress paradigm was different compared to Camp and Johnson (2015) and Lowrance *et al*. work (2016). Specifically, their stress paradigm is 6 hrs per day of restraint for 21 days. Interestingly, Conrad *et al*. did not find chronic stress to enhance fear memory. Instead, they found that stress has an interaction with metyrapone, which prevents the production of CORT. They found that chronically stressed rats given metyrapone have less fear memory compared to non-stressed rats given metyrapone. Conrad *et al*.'s work is contradicted by later studies from Conrad's laboratory where investigations (Hoffman *et al*., 2014; Hoffman *et al*., 2015) actually found that chronic stress does enhance fear memory. To further evaluate the role of CORT in facilitating fear memory formation, chronically stressed male rats and non-stressed controls were administered metyrapone prior to fear conditioning. The data demonstrate that chronic stress sensitizes memory acquisition (Figure 7) and enhances contextual fear memory when measured 24 hours after fear conditioning (Figure 8). Metyrapone fails to dampen memory acquisition

(Figure 7) but does disrupt the formation of fear memories (Figure 8). Interestingly, the two-way ANOVA failed to reveal an interaction between metyrapone and chronic stress. This indicates that the greater fear response in chronic stress animals is not dependent on an enhanced CORT response during fear conditioning.

Multiple laboratories demonstrated that the BLA was a necessary brain region for contextual fear memory following the initial fear conditioning (Maren, 1999; Gale et al., 2004; Phillips and LeDoux, 1992). In addition, Giustino and Maren (2018) found that under high stress, the locus coeruleus stimulates the BLA (and inhibits under low stress). This led us to focus CORT's role to the BLA compared to other pertinent brain regions. In addition, RU38486, a glucocorticoid receptor (GR) antagonist was utilized over mineralocorticoid receptor (MR) antagonist because Roozendaal *et al*. found that GRs facilitate memory consolidation (2009). For this reason, RU38486 was administered directly into the BLA 30 min prior to fear conditioning; however, RU38486 did not have any effect on the formation of fear memories (Figure 10). This suggests that blocking GRs was not sufficient to protect rats from fearful memories like metyrapone (CORT synthesis blocker). These data suggest that GRs in the BLA are not necessary for memory acquisition or consolidation, but GR in other brain regions such as the hippocampus could be necessary. Alternately, MR may be more important in the BLA as metyrapone would have reduced signaling at both MR and GR receptor subtypes. Unfortunately, we did not observe an enhanced freezing response in chronic stress animals in this study. We hypothesize that implantation of cannulation serves as a chronic stressor to our control rats, which resulted in control rats having altered acquisition behavior compared to the chronically stressed rats (Figure 9). Following 24 hrs after fear conditioning, the cannulated control rats had

similar contextual fear memory as the chronically stressed rats, thus we could not confidently test the role of GR in mediating the chronic stress enhancement of memory (Figure 10).

There are limitations to these studies. First, we characterized systemic CORT; however, it is still unclear the role CORT has in the BLA. Future studies could investigate GRs and MRs density in the BLA. Second, we demonstrated that chronic stress sensitizes the CORT response and enhanced contextual fear memory; however, rats that are cannulated fail to show sensitization of fear memories. This is likely due to the cannulation surgery itself causing chronic stress in the control rats. Future studies will investigate GRKO as an alternative method to block CORT's function since cannulation chronically stressed the control rats. In addition, more work will investigate the differences between RU38486 and metyrapone. Last, only GRs have been blocked when RU38486 is administered. More work is needed to determine whether MRs are important for contextual fear memory in the BLA.

Chapter 3

The Role of IL-1β on Fear Memory Article

Abstract:

Chronic stress exposure facilitates contextual fear conditioning resulting in exaggerated freezing behavior when animals are returned to the environment previously associated with an aversive stimulus. Currently, it is unclear what causes the enhanced memory at the molecular level. Here, we test the hypothesis that chronically stressed rats have enhanced IL-1β signaling in the basolateral amygdala (BLA) during fear conditioning that facilitates the formation of contextual fear memory. To answer this question, three studies were completed. First, IL-1β mRNA expression was characterized in the BLA following chronic stress and fear conditioning. Second, rats were administered various doses of IL-1β in the BLA to observe its effect on fear memory. Lastly, we investigated whether blocking IL-1β via administration of exogenous IL-1 receptor antagonist (IL-1RA), would prevent stress induced enhanced contextual fear memory. The results show that IL-1β is upregulated in the BLA following chronic stress and fear conditioning, but chronic stress does not sensitize IL-1β production in response to fear

conditioning. Direct administration of IL-1β in the BLA impairs the formation of fear memory in a dose-dependent fashion. IL-1RA has minimal effect on the formation of fear memory in control animals. Following cannulation surgery, rats fail to demonstrate chronic stress induced enhanced contextual fear memory, suggesting surgery itself results in long-term effects on the brain that mask the effects of psychological stress exposure. Collectively, the results indicate that IL-1β impairs memory formation within the BLA and chronic stress affects the regulation of IL-1β within the BLA, but it is unclear if the alternations in IL-1β regulation are responsible for the stress-induced enhancement in fear memory formation.

Introduction

Chronic stress exposure increases an organism's susceptibility to anxiety disorders, which is often characterized by increased worry, out of proportion stress, responsiveness, and avoidance of non-harmful stimuli (Eiland and McEwen, 2012). Additionally, chronic stress can enhance memory consolidation following contextual fear conditioning such that animals with prior stress exposure show exaggerated freezing behavior compared to controls when placed back into the context previously paired with foot shocks (Camp and Johnson, 2015). The exaggerated freezing behavior in stressed animals can be blocked by the administration of a beta-adrenergic receptor (β-AR) antagonist prior to fear conditioning (Camp and Johnson, 2015), but the mechanism by which β-ARs mediate enhanced memory is still unclear.

There is strong evidence that NE enhances the formation of fear memories via stimulation of β-ARs within the basolateral amygdala (BLA; Inoue *et al*., 2006). Interestingly, in control animals with no prior stressor exposure, administration of β-AR antagonists has no effect on contextual fear learning (Camp and Johnson, 2015; Kabizke, Silva, and Wiedenmayer, 2011),

suggesting normally, NE release during fear conditioning is not sufficient to stimulate the lower affinity β-ARs. The fact that propranolol, a β-AR antagonist, blocks the exaggerated freezing in rats that were chronically stressed prior to fear conditioning suggests chronic stress primes the stress response for a subsequent stressor (Camp and Johnson, 2015). This idea is supported by past studies that demonstrated chronic stress increases NE turnover within limbic brain areas including the amygdala (Porterfield *et al*., 2012), and sensitizes physiological responses to future stressors (Lowrance *et al*., 2016). In addition, β-AR signaling within the amygdala may be sensitized in chronically stressed animals as demonstrated by significantly greater IL-1β induction in stressed animals compared to controls following isoproterenol (β-AR agonist) administration (Porterfield *et al*., 2012). IL-1β is a candidate molecule to investigate since past data demonstrate IL-1β can modulate fear memory (Porterfield *et al*., 2012; Avital *et al*., 2003; Goshen *et al*., 2007; Jones *et al*., 2015; Song, Phillips, and Leonard, 2003).

Dr. Raz Yirmiya's laboratory has elegantly demonstrated that IL-1β has an inverted U-shaped dose response in affecting memory. His laboratory, and that of others have demonstrated that reductions in IL-1β signaling, such as in IL-1 receptor KO mice or following administration of the IL-1 receptor antagonist (IL-1RA), impair memory formation (Goshen *et al*., 2007; Song, Phillips, Leonard, 2003; Avital *et al*., 2003). In contrast, central administration of low concentrations of IL-1β (1ng) enhance fear memory formation, while high concentrations of IL-1β (10ng) impair memory formation (Goshen *et al*., 2007). Most research investigating the role of IL-1β on altering memory processes have focused on the effects of IL-1β within the hippocampus or the entire brain (Ben-Menachem *et al*., 2014; Jones *et al*., 2015; Goshen *et al*., 2007); however, our previous studies indicate that stress sensitizes β-AR mediated induction of IL-1β within the amygdala and not the hippocampus (Porterfield et at., 2012). Thus, the studies

presented here tested the hypothesis that prior exposure to chronic stress sensitizes the induction of IL-1β within the BLA in response to fear conditioning and that elevated IL-1β in the BLA is responsible for the enhancement in fear memory in chronically stressed rats.

To examine the role of IL-1β in the BLA we first measured IL-1β expression following fear conditioning in control and chronic stressed rats. Second, we examined a dose response of IL-1β injected into the BLA on fear memory. Lastly, we administered IL-1RA in the BLA to determine if it would be sufficient to block the stress-induced enhancement of fear memory.

Methods:

Animals and Housing: Male Fischer rats were used since this strain is highly stress responsive and more susceptible to stress-induced pathology compared to many other rat strains (Camp *et al*., 2012; Porterfield *et al*., 2011; Uchida *et al*., 2008). Adult animals (250–350 g) were single-housed in standard rat cages, and given access to food and water *ad libitum*, except when undergoing food restriction stress (see below). Rats were kept on a 12:12 h light–dark cycle (lights on at 08:00). All animals were handled according to the Animal Welfare Act and The Guide for the Care and Use of Laboratory Animals. The Kent State University Institutional Animal and Care Committee approved all procedures.

Chronic Stress Protocol: Male Fischer rats were exposed to a series of stressors following an established protocol (Camp *et al*., 2012) or were left undisturbed as home cage control animals. This four day repeated stress paradigm was chosen since we previously demonstrated that it results in increased NE turnover in the amygdala, sensitized β-AR mediated responses, and enhanced fear conditioning to contextual cues (Porterfield *et al*., 2012; Camp *et al*., 2012; Camp and Johnson, 2015). On the morning (08:00–10:00) of day 1, chronic stress rats were placed in

DecapiCone rodent restrainers (Braintree Scientific, Inc., Braintree, MA) for 60 min, before being returned to their home cage. At 15:00 h, food was removed from chronic stress rat cages for 18 h. On day 2, chronic stress rats were placed in novel habitats, containing 35 μL trimethylthiazoline (a component of fox feces) to simulate predator odor. Rats were then placed back in their home cages but were housed in constant light conditions overnight. On the morning of day 3, animals were exposed to restraint stress again for 60 min, then at 15:00 h their bedding material was dampened with approximately 1500 mL distilled water. On day 4, subjects were exposed to forced swim for 5 min in glass cylinders measuring 49 × 18.7 cm (inner height and diameter, respectively) filled approximately to the 37.5 cm line with water at a temperature of 21°C. Following this task, subjects were placed in cages containing dry bedding. Following chronic stress and prior to behavioral and physiological observations, the subjects' weights were recorded. Control rats were in their home cage for the entire duration of the chronic stress paradigm.

Fear Conditioning Paradigm: One day following the stress protocol (day 5), rats were placed in a 21.59 × 21.59 × 27.94 cm conditioning chamber (Lafayette Instrument Company, Lafayette, IN) with a floor consisting of a series of electrically conductive steel bars for a total of 5 min. At min 2 and min 4, rats received a foot shock (1.5 mA for 2 s). Following fear conditioning, rats were placed back into their home cages. Rats were recorded using a C615 HD Webcam (Logitech, Silicon Valley, CA) to measure acquisition during the time of fear conditioning. Acquisition time points were separated into "0-2 min"; "2-4 min"; and "4-5 min" for analysis of freezing behavior.

Assessment of Fear Memory: Twenty-four hours later, subjects were placed back into the conditioning chamber where behavior was recorded for 15 min and freezing behavior was evaluated. Freezing behavior was defined as complete immobility, except for movements necessary for respiration. Scoring was performed by a trained researcher blind to group assignment. Scores were obtained by checking the video every 10 sec for 15 min, and one point was assigned for each instance of freezing behavior, with a maximum possible score of 90 pts.

Tissue Collection*:* Rats were submitted to the fear conditioning paradigm on day 5 as described above. Rats were removed and returned to their home cage for 1 hr before being euthanized by rapid decapitation to collect brains to assess mRNA transcription of IL-1β in the BLA. Porterfield *et al*. found 1h after stress exposure to be ideal for measuring IL-1β mRNA following stress exposure (2011) and isoproterenol administration (2012). After decapitation, brains were extracted and frozen in a solution of isopentane and dry ice held at -20°C for approximately 60 sec. In order to remove the BLA, each brain was sliced on a cryostat until the beginning of the BLA (2.9 mm posterior to bregma). The BLA was extracted bilaterally using a blunt 18G needle to punch out the BLA. Each punch was placed in an RNase free tube and stored at -20 °C.

mRNA Extraction: Tissue was stored at -20°C before being treated with 90μL picopure extraction buffer and dissociated using a sonic dismembrator (Fisher Scientific model 100). Samples were then added to a preconditioned extraction column and allowed to incubate for 5 min at room temperature. The columns were then centrifuged at 16,000g for 2 min. After being removed from the centrifuge, 90μL of 70% ethanol was mixed with the samples. Samples were then treated with DNase for 15 min at room temperature. Following DNase treatment, the samples were washed with 40μL wash buffer 1 and centrifuged for 1 min at 8,000g. Samples

were then treated with 100µL wash buffer twice being centrifuged at 8,000g for 1 min and then 16,000g for 2 min. The purification columns were then placed on new 0.5mL microcentrifuge tubes and incubated with 15µL of RNase free water for 1 min at room temperature. The columns were centrifuged for 1 min at 1,000g to distribute the water before being centrifuged at 16,000g for 2 min to extract the mRNA from the column. mRNA concentration was then assessed using a spectrometer to measure the ng/µL in 2µL of sample and the absorption at 260/280 and 260/230. The samples were then frozen at -20°C until PCR could be performed.

cDNA Conversion and Quantitative PCR: mRNA samples were removed from the freezer and diluted to a concentration 14 ng/µL. 2µL of each corrected sample was added into a new thermocycler tube containing 18µL master mix with a 7:10:1 ratio of RNase free water, 2x RT Buffer, and 20x enzyme mix. Tubes were then ran in the thermocycler per manufacturer's instructions to convert to cDNA. Following cDNA conversion, gene expression was assessed using real time PCR with GAPDH (RN: 01775763_g1 GAPDH), IL-1β (Rn:99999009_m1IL-1b), and IL-1RA (RN00573488_1ml) taqman probes (ThermoFisher Scientific, Oakwood, OH). Master mix was prepared for all 3 gene targets using 26µL RNase free water, 31µL QPCR master mix, 3µL gene target taqman probe, and 1µL 1:500 concentration dye. For each gene target, 2µL of cDNA and 62µL of master mix were added to the tubes. From each tube, 20µL of the cDNA mixture was added to a 96 well plate in triplicate. An optical cap 8x strip was then placed on the plate and centrifuged at 4000 rpm for 1 min. The well plate was placed in the Mx2005 QPCR instrument using FAM for the target gene and ROX as the reference gene. Plates were then ran with the 1st cycle at 95°C for 3 min followed by segment 2 at 00:05 at 95°C before dropping to 60°C for 00:20 sec for a total of 45 cycles.

Cannula Implantation: Adult rats were anesthetized with 2% isoflurane and placed in a stereotaxic apparatus. A burr hole was drilled posterior to bregma using the following coordinates: [AP:−2.9mm; ML: +/- 4.5 mm lateral to the midline; DV: 8.2 mm below skull surface]. Stainless-steel guide cannula (Plastics-One Inc., VA) were secured to the skull with three stainless-steel screws, Super glue, and dental cement, and was closed by a dummy cannula (Plastics-One Inc.). Animals were administered Ketoprofen (2.0 mg/kg in 25% DMSO and 0.9% saline) immediately prior to surgery and again 24hr after surgery. Experiments were performed following 3-6 weeks of recovery. The location of the cannula was verified at the end of the study by extraction of the brain and locating the cannulation blood trail. If a cannula was found to be misplaced, the rat was excluded from the study.

Injections into BLA: In Study 6: Immediately following fear conditioning 0.5 µL of either 0.0 ng/ul (saline), 0.01 ng/ul, 0.1 ng/ul, or 1.0 ng/uL of IL-1β (R&D Systems, Minneapolis, MN) was administered into the BLA using an internal 33GA fit guide (injector) cannula with a 0.5 mm projection (Plastics-One Inc., VA). These doses were based off Goshen *et al*. work to enhance memory (2007). The injector cannula was connected to polyethene tubing (Stoelting,Wood Dale, IL) and a Hamilton 5uL syringe (Stoelting, Wood Dale, IL). Solutions were administered at a constant rate for 1 min, and the injection cannula was removed 30 sec following the termination of the injection to avoid spillage from the guide cannula. Rats were tested 24 h later for contextual fear memory, as described above.

In Study 7: Immediately following fear conditioning 0.5 ul of either saline or 25 ng/ul IL-1RA (R&D Systems, Minneapolis, MN) was administered in the BLA. This dose was based off

Borsody and Weiss work administering IL-1RA (2002). Solutions were administered at a constant rate for 1 min, and the injection cannula was removed 30 sec following the termination of the injection to avoid spillage from the guide cannula.

Statistical Analysis:

Study 5: A two-way ANOVA was used to analyze statistical significance (alpha = 0.05).
Study 6: A one-way ANOVA was used to analyze statistical significance (alpha = 0.05). A Bonferroni post hoc analysis was utilized to examine significant difference between IL-1β doses.
Study 7: A two-way ANOVA was used to analyze statistical significance (alpha = 0.05). A repeated measures analysis was utilized to analyze acquisition expression of freezing behavior (alpha = 0.05)

Results:

Study 5: The effect of chronic stress and fear conditioning on IL-1β signaling

To characterize the effect of chronic stress and fear conditioning on IL-1β signaling, IL-1β and IL-1RA mRNA was measured in the BLA 1 hr following fear conditioning. A two-way ANOVA failed to find a main effect of chronic stress [$F(1,20) = 0.541$, $p = 0.471$] but did find a significant main effect of fear conditioning [$F(1,20) = 5.570$, $p = 0.029$]. A significant interaction was observed between fear conditioning and chronic stress [$F(1,20) = 4.599$, $p = 0.044$].

IL-1RA mRNA in the BLA was also analyzed following chronic stress and fear conditioning. This was completed to more thoroughly understand IL-1β signaling in the BLA. A two-way ANOVA revealed no significant main effect of chronic stress [$F(1, 20) = 0.633$, $p = 0.436$], fear conditioning [$F(1, 20) = 0.095$, $p = 0.761$], or interaction [$F(1, 20) = 2.807$, $p = 0.109$] (Figure 12).

Study 6: The effect of IL-1β administered in BLA on fear memory

To examine the effects of IL-1β in the BLA on fear memory, IL-1β was administered directly into the BLA immediately following fear conditioning. A significant effect of IL-1β was observed [$F(3, 43) = 13.358$, $p = 0.0001$]. A Bonferroni post hoc analysis revealed a dose-dependent effect of IL-1β on fear memory with a significant decrease in percent freezing in rats administered the 0.05 ng IL-1β ($p = 0.0001$) and 0.5 ng IL-1β ($p = 0.0001$) compared to saline injected animals (Figure 13).

Study 7: The effect of chronic stress and IL-1RA administered in BLA on fear memory

To determine whether the elevation in IL-1β production during fear conditioning or following chronic stress influences the formation of fear memory, IL-1RA was administered directly into the BLA immediately following fear conditioning. The results of a repeated measures analysis revealed a significant effect of time between 0-2 min, 2-4 min, and 4-5 min [$F(1,31) = 51.308$, $p < 0.0001$) during acquisition of the fear memory (Figure 14). This suggests that the acquisition of the rats was intact. There was no significant effect of chronic stress on acquisition [$F(1,31) = 0.001$, $p = 0.970$]. A two-way ANOVA revealed no effect of stress [$F(1,31) = 0.601$, $p = 0.445$] or IL-1RA [$F(1,31) = 0.105$, $p = 0.749$] when measuring fear memory 24hr later. Moreover, there was no significant interaction [$F(1,31) = 0.339$, $p = 0.533$] (Figure 15).

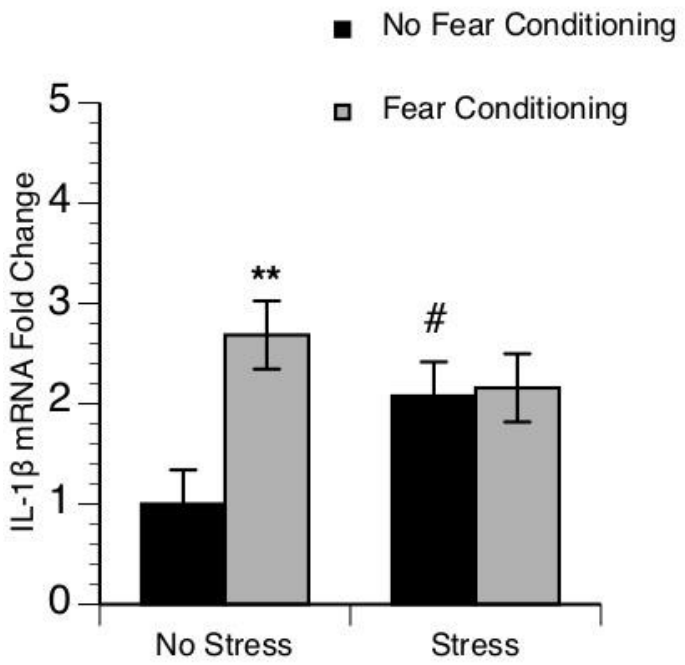

Figure 11. IL-1β mRNA is elevated in the BLA following fear conditioning: Rats were either not chronically stressed (n=12) or chronically stressed (n=12). Following, rats were separated into either no fear conditioning (n=12) or fear conditioning (n=12) treatment groups (n=6/group). All rats were placed in their home cage for 1 hour. The BLA was then collected and IL-1β mRNA was measured. # Effect of Stress $p<0.05$; ** Effect of Fear Conditioning $p<0.01$.

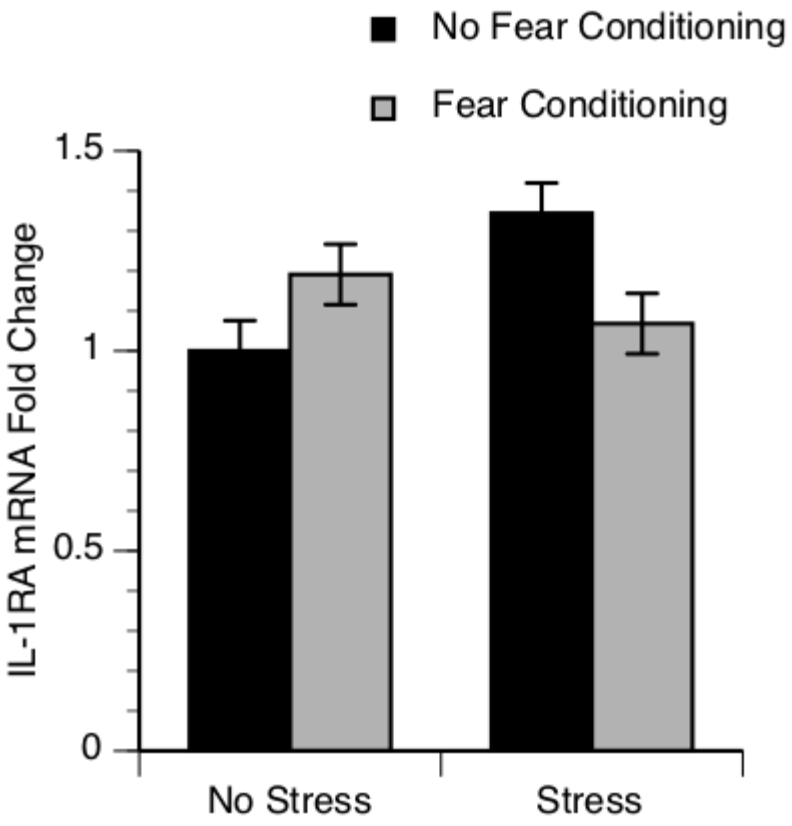

Figure 12. IL-1RA mRNA is not elevated in the BLA following fear conditioning: Rats were either not chronically stressed (n=12) or chronically stressed (n=12). Following, rats were separated into either no fear conditioning (n=12) or fear conditioning (n=12) treatment groups (n=6/group). All rats were placed in their home cage for 1 hour. The BLA was then collected and IL-1RA mRNA was measured.

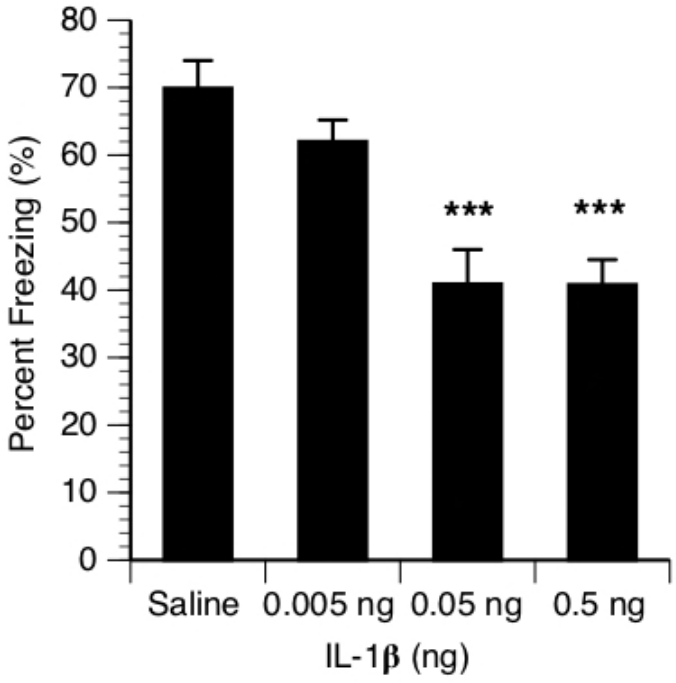

Figure 13. IL-1β administered in the BLA dampens contextual fear memory: Rats underwent a contextual fear paradigm. Immediately following fear conditioning, IL-1β or saline was administered in the BLA at the following doses: 1) Saline (n=16); 2) 0.005 ng (n=10); 3) 0.05 ng (n=10); 4) 0.5 ng (n=11) of IL-1β. Following 24 hours, rats were placed back in the same context and their percent freezing was recorded. *** $p < 0.0001$.

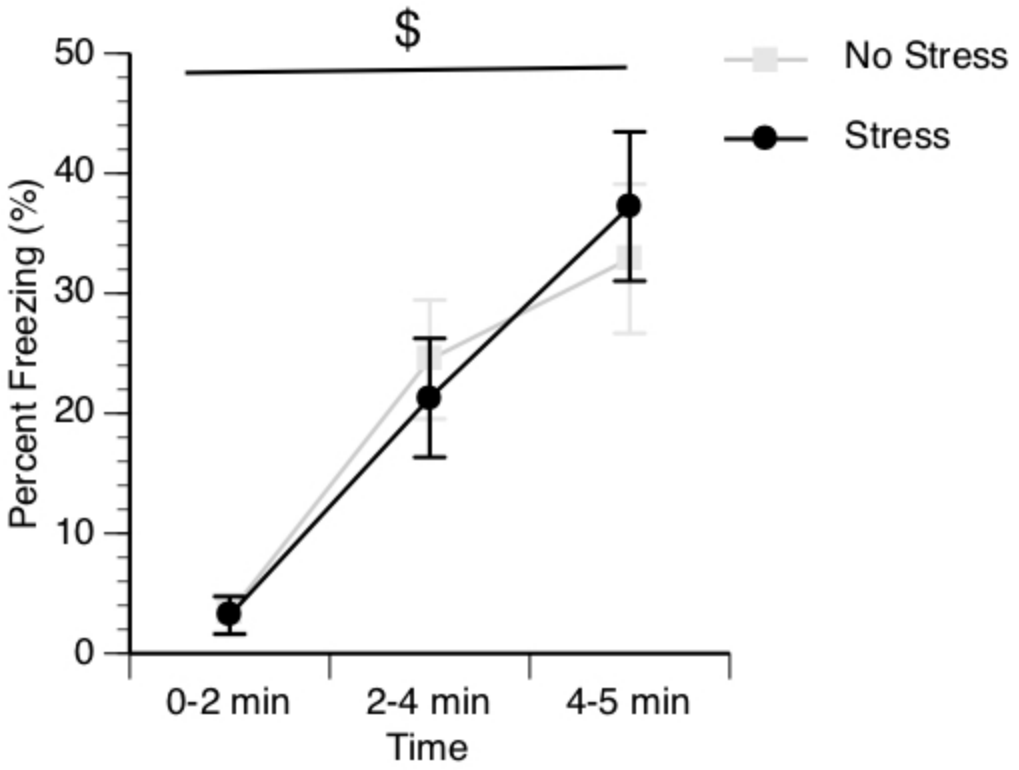

Figure 14. Cannulated rats fail to have a sensitized acquisition: Rats were either not chronically stressed (n=16) or chronically stressed (n=17). All rats underwent fear conditioning. Throughout the fear conditioning paradigm (acquisition), rats were recorded for percent freezing. The freezing was separated 0-2 min (prior to foot shock); 2-4 min (after one foot shock); 4-5 min (after two foot shocks). $ Effect of time $p<0.05$

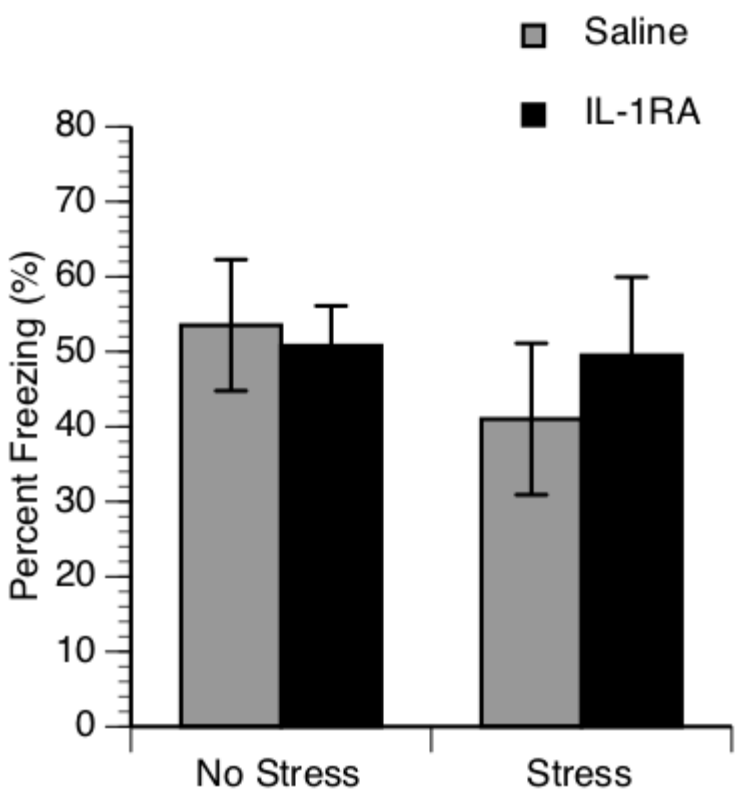

Figure 15. Chronically stressed rats failed to show enhanced fear memory: Rats were either not chronically stressed (n=16) or chronically stressed (n=16). Rats were either administered saline (n=16) or IL-1RA (n=16) immediately after fear conditioning (n=8/group). All rats underwent fear conditioning. Following 24 hrs, rats were placed in the same context and percent freezing was recorded.

Discussion:

Studies presented here investigated the role of IL-1β on contextual fear memory. It was found that IL-1β mRNA is upregulated in the BLA following fear conditioning and chronic stress; however, IL-1RA mRNA is not upregulated in the BLA following fear conditioning or chronic stress. Moreover, it was found that IL-1β administered in the BLA dampens contextual fear memory, while the administration of IL-1RA had no overall effect on memory. Surprisingly, animals implanted with cannulas failed to show a sensitized acquisition of fear memory following chronic stress.

Chronic stress sensitizes fear memory (Camp and Johnson, 2015; Jones *et al*., 2015); however, the molecular mechanism of this phenomenon was unclear. The literature suggested that IL-1β was a candidate molecule since chronically stressed rats have an enhanced production of IL-1β in the amygdala following β-AR activation (Porterfield *et al*., 2012), and central administration of low dose IL-1β has been shown to enhance fear memory (Goshen *et al*., 2007; Song, Phillips, Leonard, 2003). For this reason, this work investigated IL-1β's role in fear memory, specifically in the BLA.

The induction of IL-1 during stress exposure is dependent on stimulation of β-ARs, but a threshold level of NE release is necessary to induce IL-1 (Porterfield *et al*., 2012). Chronic stress sensitizes β-ARs mediated induction of IL-1 (Porterfield *et al*., 2012). Studies here examined whether fear conditioning itself is sufficient to induce IL-1 expression in the BLA and whether prior stress exposure would sensitize this response. Porterfield *et al*., demonstrated that chronic stress has a modulatory role in IL-1β signaling in the amygdala (2012). Specifically, when given a β-AR receptor agonist, isoproterenol, rats have normal IL-1β signaling. However, when the rats are chronically stressed, isoproterenol upregulates IL-1β signaling. Knowing this, we first

characterized the effects of chronic stress on IL-1β signaling in the BLA. In this work, we are the first to demonstrate that chronic stress upregulates IL-1β mRNA in the BLA. This compliments Porterfield *et al*.'s work since IL-1β protein increases following chronic stress in the amygdala (2012). In addition, the data revealed fear conditioning alone is sufficient to upregulate IL-1β mRNA (Figure 11). This suggests that the IL-1β in the BLA is responsive to stress and IL-1β would be elevated during memory consolidation, which supports the idea that IL-1β could modulate fear memory.

To understand the full scope of IL-1β signaling, IL-1RA mRNA was measured in the BLA following chronic stress and fear conditioning (Figure 12). This is important since Goshen *et al*. found that IL-1RA was sufficient to dampen fear memory when administered centrally (2007). In study 7, IL-1RA mRNA was not altered in either the chronic stress or fear conditioned treatment groups. This suggests that IL-1RA (IL-1 antagonist) is not being altered in the BLA 60 min following chronic stress or fear conditioning. Future studies will investigate other time points such as 24 hours as suggested in Goshen and Yirmiya's work (2009).

There is limited knowledge about IL-1β's effect on the BLA. Goshen *et al*. and Song, Phillips, Leonard found that central administration of IL-1β was sufficient to enhance fear memory (2007; 2003). However, high doses of IL-1β and IL-1RA dampen fear memory (Goshen *et al*., 2007). It was unclear where IL-1β was acting. Since the BLA is a brain region necessary for memory association following the initial fear training (Maren, 1999; Gale et al., 2004; Phillips and LeDoux, 1992), and we observed an enhancement of IL-1β signaling in the BLA, we administered IL-1β directly into the BLA of non-stressed rats. Surprisingly, IL-1β dampened fear memory at all doses tested (Figure 13). Even though this is against our initial hypothesis, there is some work that would support this finding. Specifically, Yu and Shinnick-Gallagher found that

IL-1β hyperpolarizes amygdala neurons and inhibits synaptic transmission (1994), which could be occurring in the work presented. Furthermore, we did not see a significant effect of IL-1RA administration on fear memory when injected directly into the BLA. This suggests the impairment of fear memory following intracerebroventricular injections of IL-1RA reported by Goshen *et al*. are likely due to its actions in other brain areas such as the hippocampus.

Following this knowledge, we wanted to understand the role of IL-1β on fear memory in chronic stress rats. To the best of our knowledge, the literature was limited to central administration of IL-1RA when investigating chronic stress and IL-1β signaling (Jones *et al*., 2015). Jones *et al*. found that IL-1RA administered centrally prevented chronic stress' enhancement of fear memory (2015). To determine whether this is consistent when targeting the BLA, we blocked IL-1β in stressed and non-stressed rats via IL-1RA administration in the BLA. Unfortunately, we did not observe an enhancement in fear memory in animals exposed to chronic stress (Figure 15). We hypothesize that the cannulation surgery itself masks the effect chronic stress has on fear memory. Past research, even from our own laboratory, have shown that chronic stress enhances fear memory in non-cannulated rats (Camp and Johnson, 2015; Jones *et al*., 2015). This phenomenon is likely explained by microglia being sensitized following cannulation (Holguin *et al*., 2007). As a result, the inflammatory response is already sensitized in the control rats.

This work has some limitations. First, the work only investigated mRNA levels and not protein levels. Future studies could investigate protein levels, but this limitation does not hinder the significance of the role IL-1β has on fear memories. Moreover, Porterfield *et al*. focused on IL-1β protein and found similar results (2012). Second, the data do not give information on whether IL-1β affects acquisition of the memory formation since IL-1β was given post fear

conditioning. However, the procedure was following the protocol of Goshen *et al*. to be consistent (2007). It is limited in either affecting the consolidation or retrieval of the memory since having elevated of IL-1β immediately after fear conditioning could affect behavior 24 hours later (Jones *et al*., 2015). Lastly, the cannulation surgery masked chronic stress sensitization in fear memory. Future studies will investigate new methods to target the BLA without surgery.

Chapter 4

Overall Conclusions of Dissertation

Conclusions:

Fearful memories have been evolutionary beneficial to protect organisms from learned aversive stimuli; however, when the memory activates the stress response out of proportion, the behavior is generally known as anxiety. This is financially expensive for society and damaging to clinical health. Financially, Chisholm *et al*. (2016) suggests that anxiety costs approximately 925 billion dollars per year for the world in respect to lost productivity and medical costs. Clinically, anxiety has an 18.1% of comorbidity with other behavioral disorder (Kessler *et al*., 2011). This cost to society is why the current research is crucial.

Interestingly, this cost is greater in women compared to men (Foa and Street, 2001), but the literature was limited in female rodent behavior and enhanced memories. This led us to the initial question of whether female rats are susceptible to chronic stress induced enhanced fear memory. The current data suggest female rats do not have exacerbated fear memories following chronic stress (Figure 6). This is against the initial hypothesis since women are more likely to

develop anxiety (Kessler *et al*., 2005). Moreover, these data are interesting because the data also indicate male rats are susceptible to chronic stress enhanced contextual fear memory (Figure 7; Figure 8; Camp and Johnson, 2015). It is currently unclear of the mechanism protecting female rodents.

One possible mechanism could be the CORT difference observed in female and male rats. Specifically, female rats have greater basal CORT compared to male rats (Park *et al*., 2008; Kitay, 1961). However, it was unclear whether females did not experience a sensitization of CORT following chronic stress like males do (Lowrance *et al*., 2016). We answered this question taking a baseline CORT measurement and then measure CORT following the four day chronic stress paradigm with subsequent fear conditioning and measure the change in CORT. Surprisingly, both males and females had a sensitized CORT response, which suggests that the change in CORT was not sufficient to enhance contextual fear memory. It is possible that CORT has a protective mechanism against enhanced fear memory. Interestingly, clinical data suggest that women have less CORT compared to men (Zimmer *et al*., 2003; Van Cauter *et al*., 1996; Seeman *et al*., 2001; Zhao *et al*., 2003; Kajantie and Phillips, 2006; Kudielka and Kirschbaum, 2005; Kumsta *et al*., 2007; Schoofs and Wolf, 2011), but women are more likely to develop anxiety (Kessler *et al*., 2005). On the other hand, the rodent data show females have more CORT compared to males and females do not develop chronic stress enhanced fear memory (Park *et al*., 2008; Kitay, 1961; Figure 6). In addition, Barnard *et al*. found that CORT suppresses the locus coeruleus and NE release (2019), which NE is known to enhance fear memory (Inoue *et al*., 2006).

To further understand what role CORT did have in fear memory, CORT was removed systematically via metyrapone administration prior to the contextual fear memory protocol to

determine whether CORT was necessary to develop chronic stress induced enhanced contextual fear memory. Interestingly, the data suggest that CORT is important for fear memory in general; however, there is no evidence that the sensitization of CORT is responsible for the enhancement of fear memory following chronic stress (Figure 8). This still leaves the question of where in the brain basal CORT has a modulatory role.

For this reason, the next study investigated the role CORT has in the BLA, the brain region necessary for memory association. To accomplish this, chronically stressed rats were administered RU38486 (glucocorticoid receptor antagonist) prior to fear conditioning. The results from this study are unclear because the chronically stressed rats failed to show enhanced fear memory that previous studies demonstrated. Future studies are necessary to investigate the proposed pathway in contextual fear memory. To accomplish this task, alternative methods to administer RU38486 in the BLA without cannulation is necessary. In addition, only GRs have been blocked when RU38486 is administered; more work is needed to determine whether MRs are important for contextual fear memory in the BLA.

Since the data investigating CORT was not conclusive, we extended the research to investigate a second hormone sensitive to stress, IL-1β, a known molecule to modulate fear memory. Interestingly, IL-1β has both an enhancing effect and dampening effect depending on the concentration (Goshen *et al*., 2007). However, Goshen *et al*.'s work was limited since it IL-1β was administered throughout the entire brain. To understand the role of IL-1β in fear memory, we first needed to characterize IL-1β following a stressful event. To understand the full scope of IL-1β signaling, IL-1β and IL-1RA mRNA was measured in the BLA following chronic stress and fear conditioning. This is important since Goshen *et al*. found that IL-1RA was sufficient to dampen fear memory when administered centrally (2007). IL-1RA mRNA was not altered in

either the chronic stress or fear conditioned treatment groups. This suggests that IL-1RA is not being altered in the BLA 60 min following chronic stress or fear conditioning. However, it was discovered that IL-1β was upregulated 60 min post fear conditioning. This suggests that IL-1β could play a modulatory role in the BLA. Future studies will investigate other time points such as 24 hours as suggested in Goshen and Yirmiya's work (2009). Furthermore, our work was limited to IL-1β mRNA instead of protein levels. This limitation does not hinder the significance of the role IL-1β has on fear memories. Moreover, Porterfield *et al*. focused on IL-1β protein and found similar results (2012).

It was known that IL-1β administered via I.C.V. injection into the brain was sufficient to enhance contextual fear memory, but it was unknown the location of effect in the brain (IL-1β). The next question to answer was whether administering IL-1β directly into the BLA was sufficient to enhance contextual fear memory. However, when male rats were administered with IL-1β, all doses dampened contextual fear memory. This was against the original hypothesis, but there is some evidence that explains this phenomenon. Yu and Shinnick-Gallagher discovered that IL-1β hyperpolarizes amygdala neurons and inhibits synaptic transmission (1994), which could be occurring in the work presented. In addition, Goshen *et al*. (2007) found that IL-1β can both enhance and dampen contextual fear memory depending on dosage.

Following this experiment, the role of IL-1β signaling in chronically stressed rats was still unknown. To the best of our knowledge, the literature was limited to I.C.V. administration of IL-1RA when investigating chronic stress and IL-1β signaling (Jones *et al*., 2015). Jones *et al*. discovered that IL-1RA administered centrally prevented chronic stress' enhancement of fear memory (2015). To determine whether this phenomenon is consistent when targeting the BLA, we blocked IL-1 signaling in stressed and non-stressed rats via IL-1RA administration in the

BLA. The data was not clear, similar to other cannulation studies. The data revealed that the cannulation surgery itself masks the effect chronic stress has on fear memory. Interestingly, the literature and past research demonstrated that there is no masking effect in non-cannulated rats (Jones *et al*., 2015; Camp and Johnson, 2015). In addition, we fail to see a significant effect of IL-1RA administration. This phenomenon is likely explained by microglia being sensitized following cannulation (Holguin *et al*., 2007). As a result, the inflammatory response is already sensitized in the control rats. This masking effect limits the conclusions available from the study. The data presented here also does not give information on whether IL-1β affects acquisition of memory formation. It is limited in either affecting the consolidation or retrieval of the memory. Future studies can administer IL-1β prior to fear conditioning. Lastly, the cannulation surgery masked chronic stress sensitization in fear memory. Future work is needed to block CORT and IL-1β signaling directly in the BLA without invasive surgery.

Overall, this research is impactful for the stress and fear memory literature. This work is the first to investigate potential sex differences in females on enhanced contextual fear memory. Surprisingly, females were protected from chronic stress enhanced contextual fear memory. For this reason, the subsequent studies utilized only males. In males, CORT was found to be important for normal fear memory, but sensitized CORT responses are not the mechanism underlying chronic stress induced enhancement in contextual fear memory. Interestingly, we found that IL-1β administered in the BLA impairs contextual fear memory in a dose dependent fashion. Most notably, the data presented here found that chronically stressed rats show enhanced acquisition and fear memory.

References:

Akana, S. F., Dallman, M. F., Bradbury, M. J., Scribner, K. A., Strack, A. M., & Walker, C. D. (1992). Feedback and facilitation in the adrenocortical system: unmasking facilitation by partial inhibition of the glucocorticoid response to prior stress. Endocrinology, 131(1), 57–68. https://doi.org/10.1210/endo.131.1.1319329

Avital, A., Goshen, I., Kamsler, A., Segal, M., Iverfeldt, K., Richter-Levin, G., & Yirmiya, R. (2003). Impaired interleukin-1 signaling is associated with deficits in hippocampal memory processes and neural plasticity. Hippocampus, 13(7), 826–834. https://doi.org/10.1002/hipo.10135

Barnard, D. F., Gabella, K. M., Kulp, A. C., Parker, A. D., Dugan, P. B., & Johnson, J. D. (2019). Sex differences in the regulation of brain IL-1β in response to chronic stress. Psychoneuroendocrinology, 103, 203–211. https://doi.org/10.1016/j.psyneuen.2019.01.026

Beck, K. D., & Luine, V. N. (2002). Sex differences in behavioral and neurochemical profiles after chronic stress: role of housing conditions. Physiology & Behavior, 75(5), 661–673. https://doi.org/10.1016/s0031-9384(02)00670-4

Ben-Menachem-Zidon, O., Ben-Menahem, Y., Ben-Hur, T., Yirmiya, R., & Yirmiya, R. (2014). Intra-Hippocampal Transplantation of Neural Precursor Cells with Transgenic Over-Expression of IL-1 Receptor Antagonist Rescues Memory and Neurogenesis Impairments

in an Alzheimer's Disease Model. Neuropsychopharmacology, 39(2), 401–414. https://doi.org/10.1038/npp.2013.208

Bhatnagar, S., Dallman, M. F., Roderick, R. E., Basbaum, A. I., & Taylor, B. K. (1998). The effects of prior chronic stress on cardiovascular responses to acute restraint and formalin injection. Brain Research, 797(2), 313–320. https://doi.org/10.1016/s0006-8993(98)00382-5

Blume, S. R., Freedberg, M., Vantrease, J. E., Chan, R., Padival, M., Record, M. J., … Rosenkranz, J. A. (2017). Sex- and Estrus-Dependent Differences in Rat Basolateral Amygdala. The Journal of Neuroscience, 37(44), 10567–10586. https://doi.org/10.1523/JNEUROSCI.0758-17.2017

Borsody, M. K., & Weiss, J. M. (2002). Alteration of Locus coeruleus Neuronal Activity by Interleukin-1 and the Involvement of Endogenous Corticotropin-Releasing Hormone. Neuroimmunomodulation, 10(2), 101–121. https://doi.org/10.1159/000065186

Bowman, R. E., Zrull, M. C., & Luine, V. N. (2001). Chronic restraint stress enhances radial arm maze performance in female rats. Brain Research, 904(2), 279–289. https://doi.org/10.1016/s0006-8993(01)02474-x

Buckner, J. D., Bernert, R. A., Cromer, K. R., Joiner, T. E., & Schmidt, N. B. (2008). Social anxiety and insomnia: the mediating role of depressive symptoms. Depression and Anxiety, 25(2), 124–130. https://doi.org/10.1002/da.20282

Camp, R. M., Remus, J. L., Kalburgi, S. N., Porterfield, V. M., & Johnson, J. D. (2012). Fear conditioning can contribute to behavioral changes observed in a repeated stress model. *Behavioural Brain Research*, *233*(2), 536–544. https://doi.org/10.1016/j.bbr.2012.05.040

Camp, R. M., & Johnson, J. D. (2015). Repeated stressor exposure enhances contextual fear memory in a beta-adrenergic receptor-dependent process and increases impulsivity in a non-beta receptor-dependent fashion. Physiology & Behavior, 150, 64–68. https://doi.org/10.1016/j.physbeh.2015.03.008

Chang, Y.-J., Yang, C.-H., Liang, Y.-C., Yeh, C.-M., Huang, C.-C., & Hsu, K.-S. (2009). Estrogen modulates sexually dimorphic contextual fear extinction in rats through estrogen receptor β. Hippocampus, 19(11), 1142–1150. https://doi.org/10.1002/hipo.20581

Chisholm, D., Sweeny, K., Sheehan, P., Rasmussen, B., Smit, F., Cuijpers, P., & Saxena, S. (2016). Scaling-up treatment of depression and anxiety: a global return on investment analysis. The Lancet Psychiatry, 3(5), 415–424. https://doi.org/10.1016/S2215-

0366(16)30024-4

Conrad, C. D., Mauldin-Jourdain, M. L., & Hobbs, R. J. (2001). Metyrapone reveals that previous chronic stress differentially impairs hippocampal-dependent memory. Stress (Amsterdam, Netherlands), 4(4), 305–318. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/18301732

Curzon, P., Rustay, N. R., & Browman, K. E. (2009). Cued and Contextual Fear Conditioning for Rodents. Methods of Behavior Analysis in Neuroscience. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/21204331

Dhabhar, F. S., & Mcewen, B. S. (1997). Acute Stress Enhances while Chronic Stress Suppresses Cell-Mediated Immunityin Vivo:A Potential Role for Leukocyte Trafficking. Brain, Behavior, and Immunity, 11(4), 286–306. https://doi.org/10.1006/brbi.1997.0508

Do-Monte, F. H., Quinõnes-Laracuente, K., & Quirk, G. J. (2015). A temporal shift in the circuits mediating retrieval of fear memory. *Nature*, *519*(7544), 460–463. https://doi.org/10.1038/nature14030

Donckier, J., Burrin, J. M., Ramsayl, I. D., & Joplin, G. F. (1986). Successful control of Cushing's disease in the elderly with long term metyrapone. Postgraduate Medical

Journal, 62, 727–730. https://doi.org/10.1136/pgmj.62.730.727

Eiland, L., & McEwen, B. S. (2012). Early life stress followed by subsequent adult chronic stress potentiates anxiety and blunts hippocampal structural remodeling. Hippocampus, 22(1), 82–91. https://doi.org/10.1002/hipo.20862

Faravelli, C., Lo Sauro, C., Godini, L., Lelli, L., Benni, L., Pietrini, F., … Ricca, V. (2012). Childhood stressful events, HPA axis and anxiety disorders. World Journal of Psychiatry, 2(1), 13–25. https://doi.org/10.5498/wjp.v2.i1.13

Foa, E. B., & Street, G. P. (2001). Women and traumatic events. The Journal of Clinical Psychiatry, 62 Suppl 17, 29–34. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11495093

Gale, G. D., Anagnostaras, S. G., Godsil, B. P., Mitchell, S., Nozawa, T., Sage, J. R., … Fanselow, M. S. (2004). Journal of Neuroscience. *J. Neurosci.*, *21*(10), 3619–3627. https://doi.org/10.1523/jneurosci.4100-03.2004

Giustino, T. F., & Maren, S. (2018). Noradrenergic Modulation of Fear Conditioning and Extinction. Frontiers in Behavioral Neuroscience, 12, 43. https://doi.org/10.3389/fnbeh.2018.00043

Goshen, I., Kreisel, T., Ounallah-Saad, H., Renbaum, P., Zalzstein, Y., Ben-Hur, T., … Yirmiya, R. (2007). A dual role for interleukin-1 in hippocampal-dependent memory processes. Psychoneuroendocrinology, 32(8–10), 1106–1115. https://doi.org/10.1016/j.psyneuen.2007.09.004

Goshen, I., & Yirmiya, R. (2009). Interleukin-1 (IL-1): A central regulator of stress responses. Frontiers in Neuroendocrinology, 30(1), 30–45. https://doi.org/10.1016/J.YFRNE.2008.10.001

Haleem, D. J., Kennett, G., & Curzon, G. (1988). Adaptation of female rats to stress: shift to male pattern by inhibition of corticosterone synthesis. Brain Research, 458(2), 339–347. https://doi.org/10.1016/0006-8993(88)90476-3

Heinsbroek, R. P. W., Van Haaren, F., Feenstra, M. G. P., Endert, E., & Van de Poll, N. E. (1991). Sex- and time-dependent changes in neurochemical and hormonal variables induced by predictable and unpredictable foot shock. Physiology & Behavior, 49(6), 1251–1256. https://doi.org/10.1016/0031-9384(91)90359-V

Hoffman, A. N., Parga, A., Paode, P. R., Watterson, L. R., Nikulina, E. M., Hammer, R. P., & Conrad, C. D. (2015). Chronic stress enhanced fear memories are associated with increased amygdala zif268 mRNA expression and are resistant to reconsolidation. Neurobiology of Learning and Memory, 120, 61–68.

https://doi.org/10.1016/J.NLM.2015.02.004

Hoffman, A. N., Lorson, N. G., Sanabria, F., Foster Olive, M., & Conrad, C. D. (2014). Chronic stress disrupts fear extinction and enhances amygdala and hippocampal Fos expression in an animal model of post-traumatic stress disorder. Neurobiology of Learning and Memory, 112, 139–147. https://doi.org/10.1016/j.nlm.2014.01.018

Holguin, A., Frank, M. G., Biedenkapp, J. C., Nelson, K., Lippert, D., Watkins, L. R., … Maier, S. F. (2007). Characterization of the temporo-spatial effects of chronic bilateral intrahippocampal cannulae on interleukin-1β. Journal of Neuroscience Methods, 161(2), 265–272. https://doi.org/10.1016/j.jneumeth.2006.11.014

Ihne, J. L., Fitzgerald, P. J., Hefner, K. R., & Holmes, A. (2012). Pharmacological modulation of stress-induced behavioral changes in the light/dark exploration test in male C57BL/6J mice. Neuropharmacology, 62(1), 464–473. https://doi.org/10.1016/j.neuropharm.2011.08.045

Inoue, T., Nakagawa, S., Izumi, T., Kitaichi, Y., & Koyama, T. (2006). Effect of combined treatment with noradrenaline and serotonin reuptake inhibitors on conditioned freezing. European Journal of Pharmacology, 540(1–3), 91–95. https://doi.org/10.1016/j.ejphar.2006.04.013

Janak, P. H., & Tye, K. M. (2015). From circuits to behaviour in the amygdala. Nature, 517(7534), 284–292. https://doi.org/10.1038/nature14188

Johnson, J. D., O'Connor, K. A., Deak, T., Spencer, R. L., Watkins, L. R., & Maier, S. F. (2002). Prior stressor exposure primes the HPA axis. *Psychoneuroendocrinology, 27*(3), 353–365. https://doi.org/10.1016/S0306-4530(01)00057-9

Johnson, J. D., Campisi, J., Sharkey, C. M., Kennedy, S. L., Nickerson, M., Greenwood, B. N., & Fleshner, M. (2005). Catecholamines mediate stress-induced increases in peripheral and central inflammatory cytokines. Neuroscience, 135(4), 1295–1307. https://doi.org/10.1016/j.neuroscience.2005.06.090

Jones, M. E., Lebonville, C. L., Barrus, D., & Lysle, D. T. (2015). The Role of Brain Interleukin-1 in Stress-Enhanced Fear Learning. Neuropsychopharmacology, 40(5), 1289–1296. https://doi.org/10.1038/npp.2014.317

José Jaime, H.-P., Venus, B.-C., Graciela, J.-R., Tania, H.-H. O., & Lucía, M.-M. (2016). Young-Adult Male Rats' Vulnerability to Chronic Mild Stress Is Reflected by Anxious-Like instead of Depressive-Like Behaviors. Neuroscience Journal, 2016, 1–12. https://doi.org/10.1155/2016/5317242

Kabitzke, P. A., Silva, L., & Wiedenmayer, C. (2011). Norepinephrine mediates contextual fear learning and hippocampal pCREB in juvenile rats exposed to predator odor. Neurobiology of Learning and Memory, 96(2), 166–172.

https://doi.org/10.1016/j.nlm.2011.04.003

Kajantie, E., & Phillips, D. I. W. (2006). The effects of sex and hormonal status on the physiological response to acute psychosocial stress. Psychoneuroendocrinology, 31(2), 151–178. https://doi.org/10.1016/J.PSYNEUEN.2005.07.002

Kant, G. J., Lenox, R. H., Bunnell, B. N., Mougey, E. H., Pennington, L. L., & Meyerhoff, J. L. (1983). Comparison of stress response in male and female rats: Pituitary cyclic AMP and plasma prolactin, growth hormone and corticosterone. Psychoneuroendocrinology, 8(4), 421–428. https://doi.org/10.1016/0306-4530(83)90021-5

Kelly, M. M., Tyrka, A. R., Anderson, G. M., Price, L. H., & Carpenter, L. L. (2008). Sex differences in emotional and physiological responses to the Trier Social Stress Test. Journal of Behavior Therapy and Experimental Psychiatry, 39(1), 87–98. https://doi.org/10.1016/j.jbtep.2007.02.003

Kessler, R. C., Chiu, W. T., Demler, O., Merikangas, K. R., & Walters, E. E. (2005). Prevalence, severity, and comorbidity of 12-month DSM-IV disorders in the National Comorbidity Survey Replication. Archives of General Psychiatry, 62(6), 617–627. https://doi.org/10.1001/archpsyc.62.6.617

Kessler, R. C., Berglund, P. A., Coulouvrat, C., Hajak, G., Roth, T., Shahly, V., … Walsh, J. K. (2011). Insomnia and the performance of US workers: results from the America insomnia survey. *Sleep*, *34*(9), 1161–1171. https://doi.org/10.5665/SLEEP.1230

Kishi, T., Tsumori, T., Yokota, S., & Yasui, Y. (2006). Topographical projection from the hippocampal formation to the amygdala: A combined anterograde and retrograde tracing study in the rat. The Journal of Comparative Neurology, 496(3), 349–368. https://doi.org/10.1002/cne.20919

Kitay, J. I. (1961). Sex Differences in adrenal cortical secretion in the rat. Endocrinology, 68(5), 818–824. https://doi.org/10.1210/endo-68-5-818

Kokras, N., & Dalla, C. (2014). Sex differences in animal models of psychiatric disorders. British Journal of Pharmacology, 171(20), 4595–4619. https://doi.org/10.1111/bph.12710

Kudielka, B. M., & Kirschbaum, C. (2005). Sex differences in HPA axis responses to stress: a review. Biological Psychology, 69(1), 113–132. https://doi.org/10.1016/J.BIOPSYCHO.2004.11.009

Kumsta, R., Entringer, S., Hellhammer, D. H., & Wüst, S. (2007). Cortisol and ACTH responses to psychosocial stress are modulated by corticosteroid binding globulin levels. Psychoneuroendocrinology, 32(8–10), 1153–1157.

https://doi.org/10.1016/J.PSYNEUEN.2007.08.007

LaLumiere, R. T., Buen, T.-V., & McGaugh, J. L. (2003). Post-training intra-basolateral amygdala infusions of norepinephrine enhance consolidation of memory for contextual fear conditioning. The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 23(17), 6754–6758. https://doi.org/10.1523/JNEUROSCI.23-17-06754.2003

Lebron-Milad, K., Abbs, B., Milad, M. R., Linnman, C., Rougemount-Bücking, A., Zeidan, M. A., … Goldstein, J. M. (2012). Sex differences in the neurobiology of fear conditioning and extinction: a preliminary fMRI study of shared sex differences with stress-arousal circuitry. Biology of Mood & Anxiety Disorders, 2, 7. https://doi.org/10.1186/2045-5380-2-7

Lezak, K. R., Missig, G., & Carlezon, W. A. (2017). Behavioral methods to study anxiety in rodents. Dialogues in Clinical Neuroscience, 19(2), 181–191. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/28867942

Lowrance, S. A., Ionadi, A., McKay, E., Douglas, X., & Johnson, J. D. (2016). Sympathetic nervous system contributes to enhanced corticosterone levels following chronic stress. Psychoneuroendocrinology, 68, 163–170. https://doi.org/10.1016/j.psyneuen.2016.02.027

Luine, V., Villegas, M., Martinez, C., & McEwen, B. S. (1994). Repeated stress causes reversible impairments of spatial memory performance. Brain Research, 639(1), 167–170. https://doi.org/10.1016/0006-8993(94)91778-7

Maren, S. (1999). Neurotoxic basolateral amygdala lesions impair learning and memory but not the performance of conditional fear in rats. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *19*(19), 8696–8703. https://doi.org/10.1523/JNEUROSCI.19-19-08696.1999

Matuszewich, L., Karney, J. J., Carter, S. R., Janasik, S. P., O'Brien, J. L., & Friedman, R. D. (2007). The delayed effects of chronic unpredictable stress on anxiety measures. Physiology & Behavior, 90(4), 674–681. https://doi.org/10.1016/j.physbeh.2006.12.006

McClure, E. B., Monk, C. S., Nelson, E. E., Zarahn, E., Leibenluft, E., Bilder, R. M., … Pine, D. S. (2004). A developmental examination of gender differences in brain engagement during evaluation of threat. *Biological Psychiatry*, *55*(11), 1047–1055. https://doi.org/10.1016/j.biopsych.2004.02.013

McReynolds, J. R., Holloway-Erickson, C. M., Parmar, T. U., & McIntyre, C. K. (2014). Corticosterone-induced enhancement of memory and synaptic Arc protein in the medial prefrontal cortex. Neurobiology of Learning and Memory, 112, 148–157.

https://doi.org/10.1016/j.nlm.2014.02.007

Paré, D. (2003). Role of the basolateral amygdala in memory consolidation. Progress in Neurobiology, 70(5), 409–420. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14511699

Park, C. R., Zoladz, P. R., Conrad, C. D., Fleshner, M., & Diamond, D. M. (2008). Acute predator stress impairs the consolidation and retrieval of hippocampus-dependent memory in male and female rats. Learning & Memory (Cold Spring Harbor, N.Y.), 15(4), 271–280. https://doi.org/10.1101/lm.721108

Phillips, R. G., & LeDoux, J. E. (1992). Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. *Behavioral Neuroscience*, *106*(2), 274–285. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1590953

Porterfield, V. M., Zimomra, Z. R., Caldwell, E. A., Camp, R. M., Gabella, K. M., & Johnson, J. D. (2011). Rat strain differences in restraint stress-induced brain cytokines. Neuroscience, 188, 48–54. https://doi.org/10.1016/j.neuroscience.2011.05.023

Porterfield, V. M., Gabella, K. M., Simmons, M. A., & Johnson, J. D. (2012). Repeated stressor exposure regionally enhances beta-adrenergic receptor-mediated brain IL-1β production. Brain, Behavior, and Immunity, 26(8), 1249–1255.

https://doi.org/10.1016/j.bbi.2012.08.001

Pugh, C. R., Tremblay, D., Fleshner, M., & Rudy, J. W. (1997). A selective role for corticosterone in contextual-fear conditioning. Behavioral Neuroscience, 111(3), 503–511. https://doi.org/10.1037/0735-7044.111.3.503

Roozendaal, B., Brunson, K. L., Holloway, B. L., McGaugh, J. L., & Baram, T. Z. (2002). Involvement of stress-released corticotropin-releasing hormone in the basolateral amygdala in regulating memory consolidation. Proceedings of the National Academy of Sciences, 99(21), 13908–13913. https://doi.org/10.1073/pnas.212504599

Roozendaal, B., Quirarte, G. L., & McGaugh, J. L. (2002). Glucocorticoids interact with the basolateral amygdala beta-adrenoceptor--cAMP/cAMP/PKA system in influencing memory consolidation. The European Journal of Neuroscience, 15(3), 553–560. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11876783

Roozendaal, B., McReynolds, J. R., Van der Zee, E. A., Lee, S., McGaugh, J. L., & McIntyre, C. K. (2009). Glucocorticoid effects on memory consolidation depend on functional interactions between the medial prefrontal cortex and basolateral amygdala. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *29*(45), 14299–14308. https://doi.org/10.1523/JNEUROSCI.3626-09.2009

Schoofs, D., & Wolf, O. T. (2011). Are salivary gonadal steroid concentrations influenced by acute psychosocial stress? A study using the Trier Social Stress Test (TSST). *International Journal of Psychophysiology*, *80*(1), 36–43. https://doi.org/10.1016/J.IJPSYCHO.2011.01.008

Seeman, T. E., Singer, B., Wilkinson, C. W., & McEwen, B. (2001). Gender differences in age-related changes in HPA axis reactivity. Psychoneuroendocrinology, 26(3), 225–240. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11166486

Sigalas, P. D., Garg, H., Watson, S., McAllister-Williams, R. H., & Ferrier, I. N. (2012). Metyrapone in treatment-resistant depression. Therapeutic Advances in Psychopharmacology, 2(4), 139–149. https://doi.org/10.1177/2045125312436597

Song, C., Phillips, A. G., & Leonard, B. (2003). Interleukin 1 beta enhances conditioned fear memory in rats: possible involvement of glucocorticoids. The European Journal of Neuroscience, 18(7), 1739–1743. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14622208

Staner, L. (2003). Sleep and anxiety disorders. Dialogues in Clinical Neuroscience, 5(3), 249–258. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/22033804

Uchida, S., Nishida, A., Hara, K., Kamemoto, T., Suetsugi, M., Fujimoto, M., … Watanabe, Y. (2008). Characterization of the vulnerability to repeated stress in Fischer 344 rats: possible

involvement of microRNA-mediated down-regulation of the glucocorticoid receptor. European Journal of Neuroscience, 27(9), 2250–2261. https://doi.org/10.1111/j.1460-9568.2008.06218.x

Van Cauter, E., Leproult, R., & Kupfer, D. J. (1996). Effects of gender and age on the levels and circadian rhythmicity of plasma cortisol. The Journal of Clinical Endocrinology & Metabolism, 81(7), 2468–2473. https://doi.org/10.1210/jcem.81.7.8675562

Verma, R., Balhara, Y. P. S., & Gupta, C. S. (2011). Gender differences in stress response: Role of developmental and biological determinants. Industrial Psychiatry Journal, 20(1), 4–10. https://doi.org/10.4103/0972-6748.98407

Vesga-López, O., Schneier, F., Wang, S., Heimberg, R., Liu, S.-M., Hasin, D. S., & Blanco, C. (2008). Gender differences in Generalized Anxiety Disorder: Results from the National Epidemiologic Survey on Alcohol and Related Conditions (NESARC) HHS Public Access. J Clin Psychiatry (Vol. 69). Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4765378/pdf/nihms748479.pdf

Wiemers, U. S., Schoofs, D., & Wolf, O. T. (2013). A friendly version of the Trier Social Stress Test does not activate the HPA axis in healthy men and women. *Stress*, *16*(2), 254–260. https://doi.org/10.3109/10253890.2012.714427

Yu, B., & Shinnick-Gallagher, P. (1994). Interleukin-1 beta inhibits synaptic transmission and induces membrane hyperpolarization in amygdala neurons. The Journal of Pharmacology and Experimental Therapeutics, 271(2), 590–600. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7525939

Zhao, Z.-Y., Lu, F.-H., Xie, Y., Fu, Y.-R., Bogdan, A., & Touitou, Y. (2003). Cortisol secretion in the elderly. Influence of age, sex and cardiovascular disease in a Chinese population. Steroids, 68(6), 551–555. https://doi.org/10.1016/S0039-128X(03)00083-7

Zimmer, C., Basler, H.-D., Vedder, H., & Lautenbacher, S. (n.d.). Sex differences in cortisol response to noxious stress. The Clinical Journal of Pain, 19(4), 233–239. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12840617