

**The Role of Glucocorticoid Binding During Fear Conditioning
In SPS-Induced Extinction Retention Deficits**

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Bachelor of Science in Neuroscience with Distinction.

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ABSTRACT

Single prolonged stress (SPS) is a rat model of post-traumatic stress disorder (PTSD) that mimics several symptoms of the disease in humans. Rats that are exposed to SPS typically have significant extinction retention deficits and upregulation of glucocorticoid receptors (GRs) in the hippocampus. A causal relationship between these two effects has not been established. It is possible that increased GR binding during fear conditioning is responsible for extinction retention deficits seen in this model. In order to test this hypothesis, the glucocorticoid synthesis inhibitor metyrapone was administered 90 minutes before fear conditioning. SPS rats showed higher levels of freezing during fear conditioning and failed to acquire the extinction memory at the same rate as controls. SPS rats froze more during the extinction test as well. The 25mg/kg There was no significant GR upregulation in any group. These data show that GR upregulation does not have a causal relationship with SPS-induced extinction retention deficits. It is possible that GR upregulation in SPS is protective, but this study does not provide evidence that GR upregulation plays a role in extinction learning.

Chapter 1

INTRODUCTION

Post-Traumatic Stress Disorder

Post-Traumatic Stress Disorder (PTSD) is defined by the Diagnostic and Statistical Manual, Edition 5 as a mental disorder that may develop after a traumatic event (American Psychiatric Association, 2013). It is defined by a set of symptoms including increased recursion of negative memories and emotions associated with the traumatic event, memory and mood impairments, and hyperarousal (American Psychiatric Association, 2013). While most individuals that experience a traumatic event do not develop PTSD, the estimated lifetime prevalence of the disorder in the United States is 7.8% (Kessler et al, 1995), making it a major problem in our society.

Individuals form fear memories through the process of fear conditioning. This occurs when a previously aversive stimulus, known as an unconditioned stimulus (UCS), is associated with a neutral stimulus, or a conditioned stimulus (CS). After this association, an individual responds to the CS as they would to the UCS (Maren, 2001). Extinction memories form when the CS is presented absent of the USC. A new extinction memory that the CS is not threatening in a specific context is formed while the fear memory remains intact (Bouton and Bolles, 1979).

It is thought that many of the symptoms of PTSD are due to problems in the formation of extinction memory (Norrholm et al, 2011; Peri et al, 2000, Milad et al, 2008; Quirk et al, 2006). However, it is not well understood what process in memory formation is responsible for these fear extinction memory deficits. There is some evidence that deficits in fear extinction in PTSD are due to increased formation and salience of fear memories (Norrholm et al, 2011; Peri et al, 2000). Norrholm and

colleagues (2011) showed that during a traditional fear conditioning/fear extinction paradigm, human subjects with PTSD showed greater fear-potentiated startle than healthy controls during both fear conditioning, and fear extinction, indicating enhanced fear memory acquisition and/or consolidation. It has also been found that individuals with PTSD have greater autonomic responses to fearful stimuli than healthy controls, indicating heightened levels of fear during memory acquisition (Peri, et al 2000).

To the contrary, there is evidence that PTSD patients acquire fear memory at the same rate as their healthy counterparts, but have deficits in extinction memory acquisition or consolidation (Milad et al, 2008; Quirk et al, 2006). In a study involving twins, Milad and colleagues (2008) found that the sibling with PTSD showed no differences in fear learning than their healthy siblings, but subsequently showed deficits in extinction recall. Reduced size and activity of the medial prefrontal cortex (mPFC) has been found in PTSD patients along with increased amygdala activity and decreased ability to extinguish aversively conditioned responses (Quirk et al, 2006). Being that the mPFC is involved in the fear extinction, and not the fear memory circuit (Henry et al, 2010), this gives physical evidence that the formation of extinction memories is specifically involved in PTSD (Quirk et al 2006).

In addition to mPFC abnormalities, there are many neuroanatomical and neurochemical correlates of PTSD in humans. One major finding in humans is that individuals with PTSD have smaller hippocampal volume after adjusting for age, total brain volume, and alcohol exposure (Gurvits et al, 1996). However, there are mixed findings as to whether PTSD leads to hippocampal atrophy or smaller hippocampal volume is a risk factor in the development of PTSD. In a twin study, it was found that

hippocampal volume of both the individual with PTSD and their identical twin negatively correlated with PTSD symptomatology in the affected individual, indicating that pre-trauma hippocampal volume is a risk factor (Gilbertson et al, 2002). However, differences in hippocampal volume are larger when subjects with PTSD are compared to non-trauma exposed controls than when compared to trauma-exposed controls, indicating some causal relationship (Smith, 2005). Hippocampal volume is not implicated in all instances of trauma and PTSD, and a link between PTSD and hippocampal volume is not always found (Bonne et al, 2001).

Another neurochemical marker for PTSD is the function and response to glucocorticoids, one of the main stress hormones in the body. Lower levels of cortisol were found in the urine of individuals with PTSD as well as those with a family history of PTSD (Yehuda, et al, 2000). In children, high urinary levels of cortisol and epinephrine are positively correlated with the development of acute PTSD symptoms (Delahanty et al, 2005). Similarly, higher levels of glucocorticoid receptors prior to combat exposure increases the likelihood of the development of PTSD (van Zuiden et al, 2012). Like studies involving hippocampal volume, larger differences in cortisol levels are reported when individuals with PTSD are compared to non-trauma exposed controls than trauma-exposed controls (Meewisee et al, 2007).

Many behavioral, neuroanatomical, and neurochemical correlates of PTSD in humans have been identified. However, to understand the relationship between these three markers, animal models can be used to manipulate certain factors and find the relationship between these three things.

Single Prolonged Stress

Single Prolonged Stress (SPS) is an animal model of PTSD. SPS consists of two hours of restraint stress, immediately followed by twenty minutes of forced swim, followed by a fifteen minute resting period, followed by ether exposure until general anesthesia is induced. After the stressor period, animals are given a seven day quiescent period, where they are not handled and no behavioral experiments take place (Liberzon et al, 1997; Liberzon et al, 1999; Knox et al, 2012a; Knox et al, 2012b).

SPS causes a series of behavioral and neurobiological changes that mimic specific PTSD symptoms. SPS exposed animals show increased sensitivity to the negative feedback of the hypothalamic-pituitary-adrenal (HPA) axis (Liberzon et al, 1997). One of the most notable neurological changes found was the upregulation of glucocorticoid receptors (GRs) in the hippocampus (Liberzon, et al, 1999; Knox et al, 2012b). Behaviorally, animals do not show changes in acquisition and consolidation of conditioned fear, nor to acquisition of extinction memory (Knox, et al, 2012a; Knox et al, 2012b). However, SPS exposed rats show deficits in retention of extinction memory, which will be referred to as “extinction retention deficits” (Knox et al 2012a; Knox et al 2012b).

These consistent neurobiological and behavioral changes suggest that SPS is a valid model of PTSD because of their similarity to symptoms and biomarkers of PTSD in humans. It is very well known that individuals with PTSD have increased sensitivity to negative feedback of the HPA axis (Yehuda, 2002), which is most likely partially responsible for the noted decrease in urine cortisol levels. Increased levels of glucocorticoid receptors can mimic the presence of increased GRs in humans prone to PTSD (van Zuiden et al, 2012), and allows investigators to study their role in behavior

and learning. Extinction retention deficits seen in SPS exposed animals may provide important information on how extinction deficits in humans with PTSD are formed.

Rationale for Current Study

The previously described findings suggest that the upregulation of GRs in the hippocampus may have a causal relationship with extinction retention deficits seen in the SPS model. We know that blood levels of corticosterone are elevated during fear conditioning, and because of this, there is increased GR binding in the brain. Because SPS enhances GR expression, SPS exposed rats theoretically have higher GR binding than controls during the formation of the fear memory, as there are more receptors present during a period of heightened blood corticosterone levels. This increased binding during fear conditioning may cause deficits in the consolidation of extinction memory, either by enhancing fear learning, or causing morphological changes in areas involved in extinction memory formation.

In this study, we subjected SPS-exposed and control rats to a traditional fear conditioning paradigm, where a tone (CS) was paired with a footshock (USC). Rats were given either one of two doses of metyrapone, a glucocorticoid synthesis inhibitor, or a vehicle. The next day, rats were exposed to the CS absent of the UCS in a different context, allowing an extinction memory to form. The day after, rats were tested in the extinction context by being presented with the CS absent of the UCS for extinction retention deficits. Each session began with a 210 baseline period to test whether certain groups exhibited higher levels of baseline freezing due to altered fear expression or context generalization.

We expected to see the usual extinction retention deficits in vehicle-treated SPS rats. We also expected metyrapone to attenuate extinction retention deficits in the

SPS rats, because the drug would lower the availability of blood glucocorticoids and in turn lower GR binding during fear conditioning. We expected the higher dose of metyrapone (50mg/kg) to have a higher attenuation effect than the lower dose (25mg/kg). Finally, we expected to see no effect or a smaller effect of the drug on the three control groups, as the differences in GR binding between the groups will be less dramatic.

Chapter 2

MATERIALS AND METHODS

Animals

Sixty-three male Sprague Dawley rats from Charles River (Portage, MI) were used in this experiment. Rats arrived at post-natal day 42-45, and were pair housed upon arrival. For the first two days, rats were given ad libitum access to food and water, then restricted to 23 grams of food per day for the remainder of the experiment to maintain equal weights between groups. Experimental manipulations began at least five days after arrival, at which time the rats were individually housed. All experiments were approved by the University of Delaware Institutional Animal Care and Use Committee following guidelines established by the NIH.

Behavioral Paradigm

All rats underwent a traditional fear conditioning paradigm where a tone was paired with a footshock. Fear conditioning occurred in Context A (citric acid odor, red light, fans on, transported in plastic boxes), where a 80dB 2kHz 10 second tone coterminated with a 1 second, 1mA footshock. The fear conditioning trial began with a 210 second baseline period, followed by five CS/UCS pairings and one minute inter-stimulus-intervals (ISI's). Fear extinction occurred the next day in Context B (acetic acid odor, yellow light, fans off, transported in home cages), with a 210 second baseline period and 30 CS presentations and ISI's without footshocks. Extinction testing occurred in Context B with 10 CS presentations and ISI's. All behavioral trials were recorded and measured for freezing using Anymaze software.

Experimental Timeline

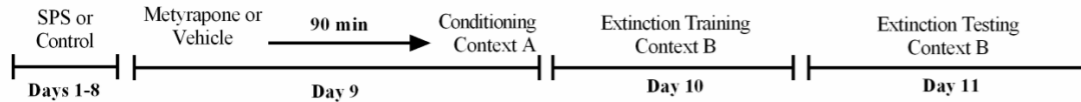


Figure 1 Experimental Timeline

The single prolonged stress procedure was performed on day 1. SPS consisted of 120 minutes of restraint stress, immediately followed by 20 minutes of forced swim, and then after a 15 minute break, exposure to ether until general anesthesia was induced. Rats were restrained in RTV 180 Tailveiner Restrainers from Braintree Scientific. Forced swim occurred with two to six rats at a time placed in an 18 gallon bucket purchased from Toys R Us filled with water at room temperature. Rats were placed on a towel under a heat lamp during the fifteen minute break. Rats were placed in a closed container with a bottom compartment filled with diethyl ether, and removed when the foot pinch reflex was absent. Control rats were kept in their home cages in an unfamiliar room during this time.

After SPS was complete, all rats were individually housed and placed back in the colony room. Rats were not handled in the quiescent period during days 2-8. On day 9, all rats underwent fear conditioning, where a tone was paired with a footshock. Ninety minutes before fear conditioning, all rats were administered the appropriate dose of metyrapone, (0, 25, or 50 mg/kg, see below). On day 10, rats underwent fear extinction, where the CS was present without the footshock. On day 11, retention of extinction was measured. On day 12, rats were euthanized and brains were collected and frozen in a -80 °C freezer to be processed on a later date.

Drug Administration

Metyrapone is a glucocorticoid synthesis inhibitor that has been used in several studies to investigate the relationship between blood circulating corticosterone levels and behavior (Blundell, et al, 2011; Barrett & Gonzalez-Lima, 2004; Roozendaal et al, 1996). Metyrapone works by inhibiting the enzyme 11-beta-hydroxylase, which is part of the chemical cascade of glucocorticoid production (Jenkins et al, 1958; Schimmer & Parker, 1996). By extension, glucocorticoid binding in the brain is kept at baseline levels, as blood-circulation glucocorticoids remain at baseline levels (Roozendaal et al, 1996).

Metyrapone was obtained from Sigma-Aldrich. Rats were randomly assigned to three drug treatment groups: vehicle, 25, and 50 mg/kg. Rats were weighed the day of fear conditioning, and drug solutions were prepared the day of administration based on that weight. Metyrapone was dissolved in a 60% saline, 40% polyethylene glycol solution, then loaded into 1 mL syringes. The drug/vehicle was then administered subcutaneously 90 minutes before the initiation of fear conditioning.

In previous studies, metyrapone has successfully modified fear and extinction learning by blocking corticosterone production in rats. Administration of 50 mg/kg of metyrapone during a water maze task impaired memory acquisition, while both 50mg/kg and 25 mg/kg impaired retention of memory (Roozendaal et al, 1996). When administered before extinction training, metyrapone inhibits formation of extinction memory (Barrett & Gonzales-Lima, 2004). This effect was repeated over a longer period of time in another study, and shown to maintain itself over time (Blundell, et al 2011).

Metyrapone is a good drug to study stress-evoked glucocorticoid release because of the lack of side effects at the appropriate doses, possibly implicating

glucocorticoid receptor binding where changes in glucocorticoid receptor expression is observed. Blundell and colleagues found that metyrapone injections at 50mg/kg and 25mg/kg have no effect on anxiety and locomotion using the elevated plus maze, open field task, light/dark task, and locomotion in a novel context (2011). They also found that the effects of metyrapone on extinction consolidation are due to lack of corticosterone production (Blundell et al, 2011). Rotllant and colleagues (2002) found that metyrapone itself can act as a stressor by increasing blood levels of ACTH, but only at 100 and 200 mg/kg, not doses less than 50 mg/kg.

Glucocorticoid Receptor Analysis

Western Blot was used to measure the glucocorticoid receptor concentration. Brains were thawed on ice and the hippocampi were removed and homogenized with homogenization buffer (50 mM Trizma base, 1 mM ethylenediaminetetraacetic acid, 10% sucrose, 4% sodium dodecyl sulfate, 2 protease inhibitor cocktail (Roche, USA pH 7.0–7.4). The samples were centrifuged at 15,000 rpm for 30 minutes and the homogenate decanted. Protein content was determined using a Pierce BCA Protein Assay Kit (Sigma–Aldrich, St. Louis, MO, USA). Approximately 20 µg of protein was then diluted into Lamelli sample buffer and stored in an 80 °C freezer until the Western Blot assay was performed.

Samples were heated for 7 min at 70 °C and, along with a molecular weight (MW) ladder (Li-COR, Lincoln, NE, USA), electrophoresed on 7.5% Tris–HCl gels (Bio-Rad Laboratories Inc., Hercules, CA, USA) and transferred onto nitrocellulose membranes. Membranes were then blocked in blocking buffer (5% powdered milk in 50mM Tris-buffered saline). Nitrocellulose membranes (0.45µm, Bio-Rad Laboratories Inc., Hercules, CA, USA) were then probed for glucocorticoid receptors

(GRs) using a rabbit polyclonal GR antibody [(1:500, Santa Cruz Biotechnology INC) 30mL PBS: 60 μ L GR] actin using a mouse β -actin antibody [(1:250, Santa Cruz Biotechnology Inc) 30mL PBS: 120 μ L β -actin) overnight at 4 °C. Nitrocellulose membranes were then rinsed and scanned in the Li-COR Odyssey Scanner in order to visualize GR and actin bands.

Statistical Analysis

Anymaze software measured freezing for all baseline, CS, and ISI time periods. For fear conditioning, the ten second CS and following 60 second ISI freezing score were averaged to give a five fear conditioning trials labeled “FC” in addition to the baseline at the beginning. For the fear extinction trials, every CS and subsequent ISI were averaged to make 30 blocks. These blocks were condensed further by averaging every two blocks to give 15 fear extinction scores labeled “FE” in addition to a baseline at the beginning. For extinction testing, each CS was averaged with the subsequent ISI were averaged to give ten extinction testing trials labeled “ET” and a baseline at the beginning.

Cued freezing during fear conditioning, extinction training, and extinction testing was separately analyzed using a stress (SPS vs. control) x drug (vehicle, 25mg/kg, 50mg/kg) x trial or block (baseline, 1-n) mixed factor design. Main and simple effects were analyzed using analysis of variance (ANOVA) while main and simple comparisons were analyzed using t-test with Bonferroni corrections applied where necessary. Comparisons were necessary for certain data points to indicate which portion of the sessions were driving the main effects. $P < .05$ was set as the threshold to define statistical significance.

Images of scanned nitrocellulose membranes were analyzed using Odyssey software (Li-COR). The integrated intensity (I.I.) of the GR and β -actin bands were expressed as a ratio (GR/Actin) and used as a relative measure of GR expression. Relative hippocampal GR levels were subjected to a 2 stress treatment x 3 drug concentration factorial design. For all statistical tests, the criterion for significance was set at $P < 0.05$.

Chapter 3

RESULTS

Fear Conditioning

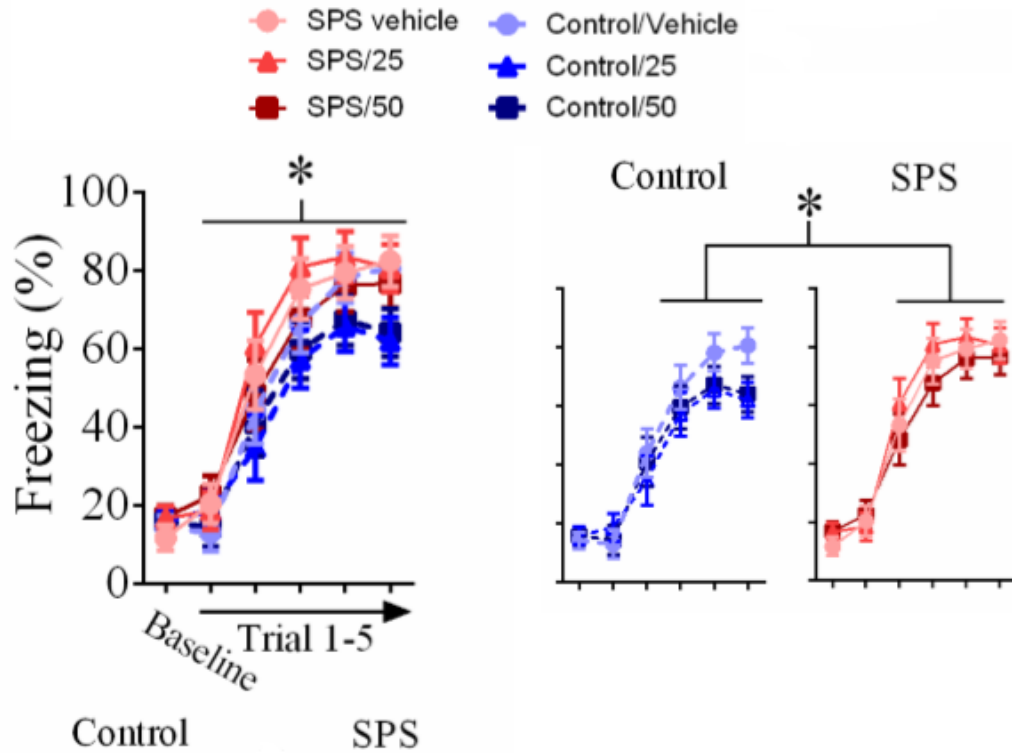


Figure 2 Fear Conditioning: ANOVA showed fear memory acquisition in all groups. SPS rats showed higher levels of freezing than controls in the last three trials.

ANOVA of cued freezing during fear conditioning revealed a main effect of trial ($F(5,260) = 215.517, p < .001$) which suggested that fear memory was acquired in all rats. SPS rats froze more during the CS presentations of the fear conditioning session, and this effect was most pronounced towards the end of the fear conditioning

($F(15,930) = 46.431, p < .001$) and a significant main effect of blocked-trial on the quadratic trend component ($F(1,62) = 24.645, p < .001$). These findings suggest that all rats expressed cued fear memory and acquired cued extinction memory. There was a main effect of stress ($F(1,62) = 4.992, p = .029$), which suggested that cued freezing was higher in SPS rats when compared to control rats during the extinction training session.

It did appear however, that SPS rats differed mostly from controls in freezing in last few extinction training trials. In order to better identify components of the extinction training session in which SPS enhanced cued freezing, we conducted further analyses. We subjected baseline freezing of the extinction training session to a t-test (SPS vs. control) to determine if SPS disrupted contextual fear memory discrimination. Baseline freezing between SPS and control rats was not statistically different ($t(66) = .297, p = .767$), which suggests SPS had no effects on contextual fear memory discrimination. Next, we subjected CS-induced freezing during the first two blocks of the extinction training session (i.e. four CS presentations) to a stress x trial factor design in order to determine if SPS enhanced cued fear memory retrieval. ANOVA of CS-induced freezing during the first two blocks of the extinction training session did not reveal any main ($F(1,62) = 1.238, p = .270$) or interaction effects ($F(2,62) = .802, p = .667$) of stress, though there was a main effect of trial ($F(1,62) = 64.243, p < .001$), which reflected an increase in cued freezing from block 1 to block 2. Lastly, we subjected cued freezing during the last block of the extinction training session (i.e. last two CS presentations) to a t-test (SPS vs. control). This comparison was statistically significant ($t(66) = 2.354, p = .022$), which suggest SPS disrupted acquisition of cued fear extinction memory.

Extinction Testing

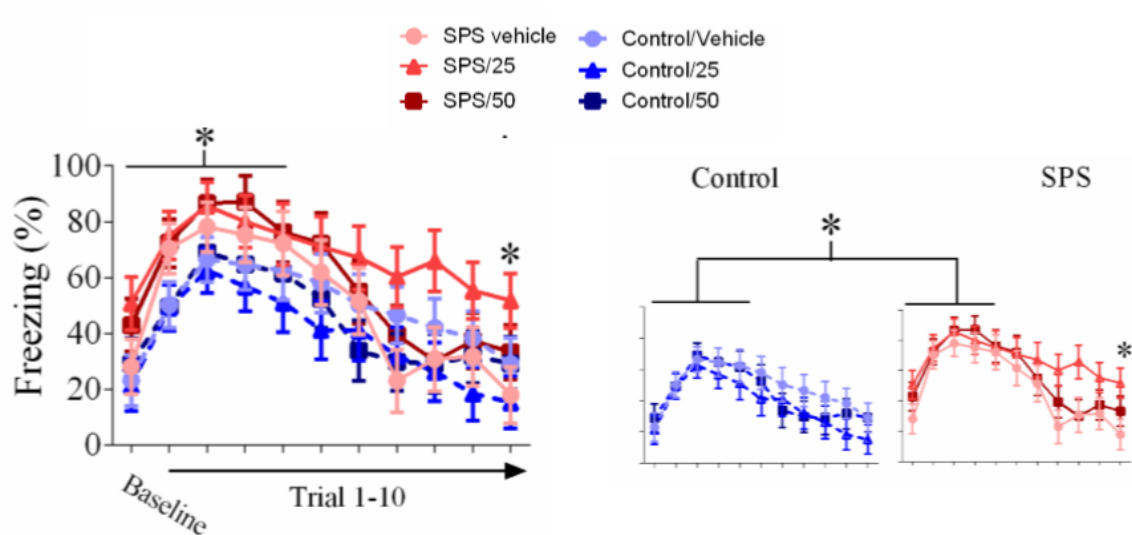


Figure 4 Extinction Retention Testing: SPS rats showed higher levels of freezing during the beginning of the extinction testing session. Freezing of the SPS 25 mg/kg group was enhanced at the end of the session.

Figure 4 shows the results of extinction retention testing for all groups.

ANOVA of cued freezing during the extinction test revealed a main effect of stress ($F(1,52) = 5.152, p = .027$) and a stress x drug x trial interaction on the linear trend component ($F(2,62) = 3.165, p = .049$). These analyses suggest that SPS enhanced cued freezing during the extinction test and that metyrapone administration prior to fear conditioning had differential effects on cued freezing in SPS and control rats. To explore these differences further, we first subjected baseline freezing to a stress x drug factor design. ANOVA revealed a significant main effect of stress ($F(1,62) = 4.422, p = .04$), but no main ($F(1,62) = .802, p = .45$) or interaction effects ($F(2,62) = .881, p = .45$).

= .419) of drug. This analysis suggests that SPS rats developed contextual fear conditioning to the extinction context even though this context was never paired with footshocks.

Next, we subjected CS-induced freezing during the first four CS presentations of the extinction test to a stress x drug x trial factor design. ANOVA revealed a main effect of stress ($F(1,62) = 8.239, p = .006$), but no main effects ($F(1,62) = 0.219, p = .642$) or interaction effects of drug ($F(1,62) = 3.250, p = 0.079$). This analysis suggests that deficits in acquisition of cued fear extinction memory persisted into the extinction test.

Lastly, we subjected cued freezing during the last trial of the extinction test to a stress x drug factor design. There was a significant stress x drug interaction ($F(2,62) = 3.215, p = .047$). Furthermore, cued freezing in the SPS/25mg/kg group was enhanced when compared to the SPS/vehicle group ($t(19) = 2.848, p = .02$). Indeed, cued freezing in the SPS/25mg/kg group was higher than all other groups in the last trial of the extinction test. This drug effect was limited to SPS rats since cued freezing in the control/25mg/kg group was not significantly different from the control/vehicle group ($t(12) = 1.12, p = .55$), though cued freezing during the last trial was attenuated in the control/25mg/kg group relative to the control/vehicle group. The 50mg/kg dose of metyrapone had no effect in SPS ($t(19) = 1.12, p = 0.28$) or control rats ($t(22) = 0.016, p = 0.99$). These analyses suggest that cued fear extinction memory deficits induced by SPS were exacerbated in SPS rats that received 25mg/kg of metyrapone prior to fear conditioning.

Western Blot

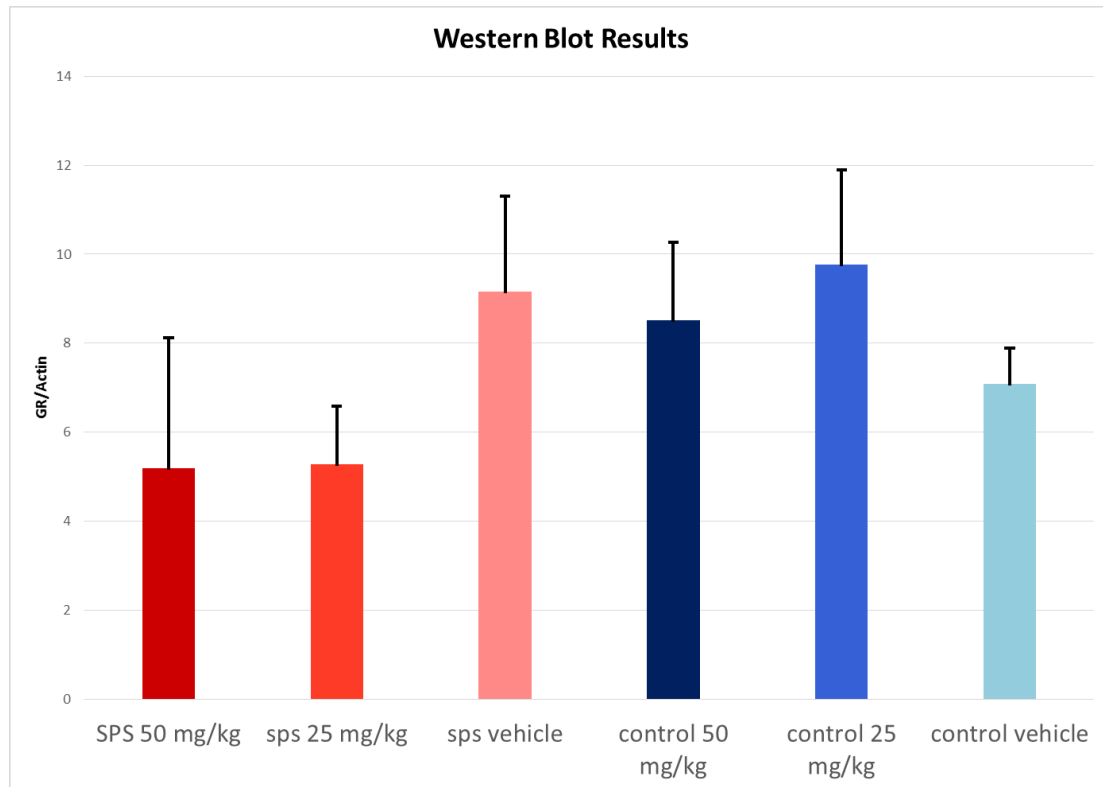


Figure 5 Western Blot: There was no significant upregulation of glucocorticoid receptors in any of the groups.

Figure 5 shows the GR/Actin ratio for all groups. No significant glucocorticoid upregulation was observed in any group. A 2x3 factorial design revealed no main effects of stress ($F(1,66) = 1.010$, $p = .319$), and drug ($F(2,66) = .086$, $p = .918$), nor any interaction effect ($F(2,66) = 2.175$, $p = .122$). While mean GR/Actin ratios were numerically higher in some groups (for example, control 25 mg/kg versus SPS 25 mg/kg), large error in every group obscures any meaningful interpretation of these differences.

Chapter 4

DISCUSSION

Overall, administration of 25mg/kg of metyrapone during fear conditioning increased the extinction retention deficits seen in SPS exposed rats. Though not significantly, the 25mg/kg dose of metyrapone during fear conditioning increased the retention of extinction memory in control rats. SPS rats also showed higher expression of fear during the fear conditioning session. Most importantly, there were no differences in hippocampal GR levels. Given the unexpected results of both the behavioral and molecular data, it is difficult to attribute these behavioral changes to any specific glucocorticoid receptor binding in the brain.

Extinction Retention Deficits: A Mechanism

As mentioned previously, there is no current agreed upon mechanism for the extinction deficits seen in PTSD patients. In most previous studies involving SPS, there were no differences in freezing behavior between SPS and control rats in the fear conditioning and extinction training trials, only the extinction testing trials. This supports the notion that extinction retention deficits in SPS rats arise from problems in consolidating extinction memories.

In this study, however, SPS rats froze at higher rates in all three behavioral tests. Higher freezing during fear conditioning may be an indication of higher acquisition of fear. If this were the case, it would be consistent with the fact that SPS rats showed deficits in the acquisition of the extinction memory, which can be a sign of a more salient fear memory. From this particular experiment, it is tempting to conclude that extinction retention deficits in SPS are caused by enhanced acquisition of fear memories.

There are several problems with this assertion. First, this is the first of many studies involving SPS where SPS rats show higher levels of freezing in the fear conditioning and fear extinction sessions (Knox et al, 2012a; Knox et al, 2012b; Keller et al, 2015). Given this history, it is unlikely that this effect will be repeated. Secondly, it is worth noting that in most fear conditioning sessions involving SPS, all groups are close to ceiling levels of freezing towards the end of the session. In this study, control 25 mg/kg and control 50 mg/kg groups are freezing at about 60% during the last trial. It is more likely that this effect is derived from lower levels of fear expression in metyrapone administered control rats. More experimentation is needed to repeat this effect and determine whether lower levels of circulating corticosterone in healthy animals disrupts fear acquisition or simply alters fear expression (i.e. differences in locomotor activity and freezing).

Besides previous studies that directly indicate extinction memory consolidation as the cause for extinction retention deficits seen in SPS rats (Knox et al, 2012a; Knox et al, 2012b; Keller et al, 2015), there is further evidence to support this hypothesis. While not displayed as consistently as in the hippocampus, glucocorticoid receptor upregulation has been observed in the prefrontal cortex (Knox et al, 2012b). Being that the prefrontal cortex is an important structure in the fear extinction circuit (Henry et al, 2010), this alteration is a possible cause of extinction retention deficits seen in SPS, and can be tested by measuring GRs in the prefrontal cortex.

Disassociating GR Upregulation and Extinction Retention Deficits

This is the first of several studies involving SPS that did not find significant GR upregulation in the hippocampus (Liberzon et al, 1999; Knox et al, 2012a; Knox et al, 2012b). Considering that extinction retention deficits were still displayed in the

SPS vehicle rats, the possible causal relationship between increased GR binding and extinction retention deficits is less likely. It is much more likely that there is one common causal factor in the development of extinction retention deficits and glucocorticoid receptor upregulation. It is also safe to say that the upregulation of GRs is not necessary or guaranteed.

There is further evidence that GR binding and extinction retention deficits are not directly linked. In a study involving both male and female rats, only male rats displayed extinction retention deficits following SPS. However, female, not male rats displayed significant GR upregulation in the hippocampus. While the lack of GR upregulation could be attributed to slight differences in the experimental manipulation, GR upregulation was neither a necessary nor sufficient condition for extinction retention deficits (Keller et al, 2015).

Significant GR upregulation in the hippocampus has been observed in SPS alterations without the presence extinction retention deficits. Knox and colleagues (2012b) found significant glucocorticoid receptor upregulation in the hippocampus after exposing rats to partial SPS treatments, where one of the three stressors of SPS was removed. Since extinction retention deficits were only present in the complete SPS group, and the complete SPS group showed significantly higher GR upregulation than the partial SPS group, the authors hypothesized that a threshold level of GR upregulation was required for extinction retention deficits to occur (Knox et al, 2012b). However, the current study and the described female SPS study (Keller et al, 2015) contradict this hypothesis.

Multiple Targets of Metyrapone

One of the major problems with studying glucocorticoid binding using the drug metyrapone is the fact that this drug indirectly targets multiple areas and receptors. Metyrapone was used to study inhibition of GR binding because of the lack of direct antagonists that cross the blood brain barrier. The effects of stereotaxic surgery and anesthesia on extinction retention deficits and glucocorticoid receptor upregulation are also unknown. Even if glucocorticoid receptor upregulation in the hippocampus was observed as it was in the past, it would still be difficult to draw conclusions about GR binding without further investigation. Metyrapone's antagonistic effects are systemic as inhibition of glucocorticoid synthesis prevents increased GR binding in the entire body, not just certain targets in the brain.

One major alternative target to consider is the mineralocorticoid receptor (MR), which is known to be located in clusters in the hippocampus (Arriza et al, 1988). Mineralocorticoid receptors have a much higher binding affinity to natural glucocorticoids like corticosterone and cortisol, leaving them occupied in most cases. Considering that metyrapone does not lessen blood glucocorticoid levels below baseline levels (Roozendaal et al, 1996) and that mineralocorticoid receptors are occupied at baseline (Lowy, 1989), it seemed unlikely that MRs could be involved in the behavioral changes observed.

However, this study did not rule out MR antagonism by metyrapone, which may explain the complete lack of effects at the higher doses of metyrapone. There is some evidence that higher levels of metyrapone administration can affect mineralocorticoid receptor binding. MRs have been shown to be important in the formation of certain types of memories. Roozendaal and colleagues (1996) found that the higher dose of metyrapone (50 mg/kg) caused rats to behave inappropriately in

certain tests (i.e. spend less time freezing in a previously stressful context), which is consistent with the memory impairments observed when blocking MR binding. While there is no direct evidence of blocked MR binding in the current study, this is a possible explanation for lower freezing in the 50mg/kg group than the 25mg/kg group. This along with the lack of positive results suggests that 50 mg/kg may not be an ideal dose to study GR activation specifically, however the cause of these negative results is still unknown.

Altered Functionality of GR Binding and Fear Memory Processes

It is still unknown how fear memory processes are affected by altered levels of circulating glucocorticoids. It is difficult to use circulating glucocorticoids to study any particular receptor in any particular location because of their non-specificity in binding and behavioral/neurochemical changes.

In control rats, though the data is not statistically significant, the actions of metyrapone are actually consistent with previous studies. Inhibition of corticosterone has been shown to inhibit consolidation of certain memories (Roosendaal et al, 1996; Barrett & Gonzales-Lima, 2004; Blundell et al, 2012). This could possibly explain the lower levels of freezing seen in control rats during fear conditioning and extinction training.

What has not been seen before is the reaction of SPS rats to inhibition of circulating corticosterone. It appears as though SPS rats have the opposite reaction to circulating corticosterone to that of healthy control rats. One possible explanation is that under times of high stress, the functionality of glucocorticoid binding becomes adaptive. High glucocorticoid release during the stressor (i.e SPS) may have caused this change to occur.

It is also possible that something about SPS changes the functionality of the extinction circuit through glucocorticoid receptor upregulation in the hippocampus and/or prefrontal cortex. The circuits may be less effective due to their receptor profile. It is also possible that the GR upregulation occurred to compensate for the decreased effectiveness and activity of the extinction circuit. Direct infusion of agonists/antagonists into the appropriate brain regions (possibly all regions of the extinction circuit) is necessary to elucidate these circuit changes.

Glucocorticoid Receptor Binding in SPS: Future Directions

The current data indicates that finding a causal relationship between hippocampal glucocorticoid receptor binding during fear conditioning and extinction retention deficits is unlikely. However, there is still good reason for exploring the effects of glucocorticoid receptor binding in SPS. The role of increased hippocampal glucocorticoid receptor binding in SPS has never been directly addressed. Direct infusion of GR agonists/antagonists into the hippocampus would need to be used to explore their direct behavioral effects. Given the fact that prefrontal cortex GRs are sometimes elevated (Knox et al, 2012b), drug infusions should be explored in this region as well.

Following this logic, one of the first tasks for researchers would be to see if surgery and SPS can be performed together. One obvious challenge would be to alter the SPS procedure to allow cannulae placements in SPS-exposed rats. This would eliminate the possibility of GR binding in other areas of the brain and body to be responsible for SPS induced behavioral changes. As it is currently performed, a rat with a surgically implanted device on its head would not fit in the restraints. Preventing the water from the forced swim from contaminating the cannula and, by

extension, the brain, is also a major concern. The effects of surgery on SPS induced behavioral and histological changes are also unknown. It is possible that stress from surgery may mask SPS induced extinction retention deficits. The combination of SPS and surgical stress may introduce undesired behavioral changes in the experiment. It is also not known how anesthetics used in surgery (i.e. isoflurane) will change the receptor profile in both SPS and control animals, especially as it relates to glucocorticoids.

It is also possible that GR receptor binding during a different stage in the experiment (i.e. extinction training) is partially responsible for the extinction retention deficits seen in SPS. The higher numbers of GRs in the hippocampus is present through all three days of behavioral experiments, so GR binding during the extinction training and testing is altered as well. This particular experimental setup would not be appropriate to test the effects of GR binding during extinction in SPS, as it has already been shown that metyrapone administration prior to extinction training inhibits extinction learning in healthy mice (Blundell et al, 2012), this can be tested by directly infusing GR agonists and antagonists directly into the hippocampus and prefrontal cortex. If extinction circuits are functionally altered as previously described, activation/inactivation of these areas during extinction training may ameliorate extinction retention deficits in SPS rats.

Conclusion

A causal relationship between glucocorticoid receptor binding in the hippocampus and extinction retention deficits in the single prolonged stress model is not supported by the data in this study. Alternate mechanisms of both glucocorticoid

receptor upregulation and extinction retention deficits in single prolonged stress must be explored.

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