Glucocorticoid Effects on Learning, Memory, and CRF

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Arts at George Mason University

by

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DEDICATION

This is dedicated to my family.
ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Jane Flinn, for her advice and support throughout my degree. To my committee, Dr. Jane Flinn, Dr. Sue Bachus, and Dr. Bob Smith, your expertise and help in this thesis has been truly appreciated. I have learned many things in and out of the lab by looking up to you all. I would also like to thank Katelyn Boggs and Stephen Lippi who helped make this happen.
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<td>Corticosterone</td>
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<td>PND</td>
<td>Postnatal Day</td>
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<td>SHRP</td>
<td>Stress Hyporesponsive Period</td>
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<td>CS</td>
<td>Conditioned Stimulus</td>
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<td>US</td>
<td>Unconditioned Stimulus</td>
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<td>CR</td>
<td>Conditioned Response</td>
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<td>IL</td>
<td>Infralimbic Cortex</td>
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<td>LA</td>
<td>Lateral Amygdala</td>
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<td>CE</td>
<td>Central Nucleus of the Amygdala</td>
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<td>HPA</td>
<td>Hypothalamic-Pituitary-Adrenal</td>
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<td>CRF</td>
<td>Corticotropic releasing factor</td>
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<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
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ABSTRACT

GLUCOCORTICOID EFFECTS ON LEARNING, MEMORY, AND CRF
Kevin Schmidt, M.A.
George Mason University, 2014
Thesis Advisor: Dr. Jane Flinn

Glucocorticoids are naturally circulating stress hormones that are also commonly synthesized and administered for medical treatments. Corticosterone (CORT) is the primary rat glucocorticoid and recent research has shown that treatments with this steroid can have both beneficial and detrimental effects on learning- and memory-related behaviors, brain structures, and the corticotropic releasing factor (CRF) chemical system. The present experiment administered CORT injections twice daily in rats on postnatal days 15-17 at three different doses: 0.04 mg/g CORT, 0.02 mg/g CORT, and 0.005 ml/g oil vehicle alone. Animals were tested on trace fear conditioning on postnatal day 28, extinction on day 29, and extinction recall on day 30. All animals conditioned and extinguished but there were no significant differences between groups. An in situ hybridization assay found that 0.02 mg/g CORT caused significant elevation in CRF mRNA expression in the infralimbic (IL) cortical region compared to control and 0.04 mg/g CORT while the lateral amygdala was not affected. Thus, CORT has in inverted-u
relationship on CRH mRNA regulation in the IL but not the LA and is not correlated with the acquisition of trace fear conditioning.
CHAPTER ONE: INTRODUCTION

**Glucocorticoids**

The vertebrate hypothalamic-pituitary-adrenal axis (HPA axis) is part of the neuroendocrine system that controls reactions to stress while regulating bodily processes such as digestion and immune function (Selye, 1975; Selye, 1976; Tsigos & Chrousos, 2002). In reaction to a stressor, corticotropic releasing factor (CRF) is released from the paraventricular nucleus of the hypothalamus. From there, CRF binds to receptors on the anterior pituitary causing a release of adrenocorticotropic hormone (ACTH). ACTH is released into the bloodstream with its primary region of action at the zona fasciculata within the cortex of the adrenal glands, which in turn causes release of glucocorticoids into the bloodstream (Sapolsky, Romero, & Munck, 2000; Ulrich-Lai & Herman, 2009). Glucocorticoids are a class of naturally circulating steroid hormones which allow the organism to adapt and cope with the stressor (Wolf, 2003). Circulating glucocorticoids have a Yerkes-Dodson relationship with cognitive performance; extreme concentrations of glucocorticoids impair cognitive abilities, while moderate levels tend to facilitate learning & memory formation (Mateo, 2008; Pavlides, Watanabe, & McEwen, 2004). In addition to concentration, the length of exposure to glucocorticoids has an effect on cognition: acute exposure tends to facilitate training, whereas chronic exposure impairs the learning process (De Kloet, et al., 1998; De Kloet, Oitzl, & Joels, 1999; Sandi, Loscertales, & Guaza, 1997).
**Glucocorticoid Medical Treatments**
Modern medical treatments commonly administer synthetic glucocorticoids to pregnant mothers to increase fetal lung maturation before a premature birth, to young children for asthma treatment, and to older adults to attenuate respiratory illness (Andrews & Matthews, 2003; Bender, Lerner, & Kollasch, 1998; Bender, Lerner, & Poland, 1991; Moritz, Cuffè, & Singh, 2012; Nieman, 2014). Recently, such treatments have been found to have harmful side effects in humans and animals. Murine studies that expose the fetus to synthetic glucocorticoids have shown that these treatments can permanently alter the behavior and stress response of the offspring that continues through development (Mathews, 2002). Human studies investigating glucocorticoid treatments such as beclomethasone dipropionate inhalants for asthma in children (6-13 year olds) have demonstrated developmental effects such as an inhibition of growth and functional suppression of the HPA axis (Nieman, 2014; Wolthers & Pedersen, 1991). Research on human males in their twenties additionally found that hydrocortisone treatment can cause deficits in emotionally laden learning & memory systems (Henckens, et al., 2011; Kirschbaum, et al., 1996). Because of these findings, more animal models have been developed to further investigate the cause of these learning & memory treatment effects.

**Learning & memory**
In animal models, a task used to study this learning & memory system is associative trace conditioning (Gormezano, Kehoe, & Marshall, 1983; Lavond, Kim & Thompson, 1993). This type of paradigm involves the formation of an association between an initially neutral tone stimulus (CS) and a mild electrical foot shock (US) that elicits a freezing response. Freezing is a fearful behavioral arrest elicited when a prey
animal feels threatened. With training, the learned pairing of the CS and US will result in a freezing reaction to the CS in the absence of the US. Specifically, trace conditioning is characterized by a separation in time between the CS and US, forming a temporal gap during which a memory trace of the CS must be maintained in order for the association to form (Han, et al., 2003). Numerous experiments have determined that the hippocampus is essential to properly acquire the conditioned response in trace conditioning (Kim, Clark, & Thompson, 1995; Moyer, Deyo, & Disterhoft, 1990; Solomon, et al., 1986). Lesions to the hippocampus of adult rabbits significantly impaired trace but not delay conditioning relative to the controls, and hippocampal stimulation has been shown to improve learning in trace conditioning (Prokasy, Kesner, & Calder, 1983; Weiss, et al., 1999).

This type of memory paradigm can also be used to investigate the extinction of these learned associations. Extinction is different from forgetting because the individual learns that the CS ceases to predict a US. Due to this new learning, the manifestation of the CR is inhibited but the memory for the association between the CS and the US is not erased (Bouton & King, 1983; Pavlov, 1927; Quirk, 2002). After the CS and US are paired in conditioning, such that the sound of the CS alone causes a freezing response, the time it takes to extinguish the CS-US association can also be measured. The CS alone can be presented for a number of trials to measure how long it takes to form a new association where the CS is no longer associated with the US.

The extinction of fearful memories involves interactions between the medial prefrontal cortex (mPFC) and the amygdala (Akirav & Maroun, 2007). Lesions of the amygdala in humans and other animals cause impaired learning of conditioned emotional
responses (Bechara, et al., 1995; LaBar, et al., 1995). Growing evidence also suggests that the mPFC plays an important role in regulating the HPA response to emotionally stressful learning by moderating the convergence of emotionally relevant information in the amygdala (Bush, Luu, & Posner, 2000; Bush, et al., 1998; Diorio, Viau, & Meaney, 1993; Figueiredo, et al., 2003; Kerns, et al., 2004; MacDonald III, et al., 2000; Spencer, Buller, & Day, 2005). Lesions in the rat mPFC impair extinction, stimulation of this region inhibits learned emotions, and extinction paradigms cause increased activation in this area of the brain (Quirk, et al., 2000; Milad, Vidal-Gonzalez, & Quirk, 2004; Santini et al., 2004).

**Side Effects of Treatment in the Brain**

Using these animal models, researchers have been able to focus on specific anatomical pathways implicated in CORT effects on learning & memory. Glucocorticoids can have an effect on learning & memory through specific bidirectional interactions between the amygdala and mPFC (Roozendaal, et al., 2009). Research has shown that connections between the infralimbic region of the mPFC (IL), the lateral nucleus of the amygdala (LA), and central nucleus of the amygdala (CE) serve as a locus for some of the physical changes responsible for classical conditioning (Berretta, et al., 2005). Projections from the IL inhibit the activation of the LA during extinction. The LA also connects to the CE which mediates the autonomic response from the HPA axis through hypothalamic connections during extinction paradigms (Akirav & Maroun, 2007; Davis, 1992; Milad, et al., 2004; Pare, et al., 2004). Acute CORT injections directly into the IL can lead to a reduction in cFOS expression (a reduction of neural activity) in this
region which also parallels an inhibition of decision making (Koot, et al., 2013; Koot, et al., 2014). Interestingly, there is a relationship with CORT in this area where 0.025 mg/g subcutaneous CORT treatments leads to a decrease in dendritic length in layers 2 and 3 of the IL compared to control treatments and adrenalectomy also leads to a decrease in total dendritic length (Cerqueira, et al., 2007). In further studies, atrophy of the dendrites in the IL peaked at about 6 days following CORT treatment (Kim, et al., 2014). Experiments investigating CORT effects on the LA have shown that acute treatment of CORT can cause strong hypertrophy of dendrites in the LA which peak around 12 days following administration (Kim, et al., 2014). In another study, 3 week CORT administration did not have significant effects on dendritic remodeling in the LA (Morales-Medina, et al., 2009). These investigations have led to conflicting conclusions regarding CORT effects on the LA.

**CRF**

CRF is one of the first signals in the stress response cascade and has a dynamic relationship with glucocorticoids and learning & memory. CRF plays a more complex role in brain signaling than just pituitary ACTH activation and one of the side effects of CORT treatment is the modulation of this chemical system. Overexpression of the CRF gene in transgenic mice causes increased production of ACTH and CORT which develops into Cushing’s syndrome symptomology, such as memory dysfunction (Bale & Vale, 2004; Stenzel-Poore & Cameron, 1992). Another mouse strain completely lacking the CRF gene exhibits reduced baseline levels of CORT and a blunted stress response (Bale & Vale, 2004; Muglia, et al., 1995).
An abundance of CRF receptors are found in the LA of rats and are post synaptic to CRF-containing projections from IL (Chalmers, Lovenberg, & De Souza, 1995; Van Pett, et al., 2000). A number of studies have demonstrated the role of CRF receptor containing neurons of the LA in emotionally charged, amygdalar-dependent learning & memory formation (Deak, et al., 1999; Hubbard, et al., 2007; Roozendaal, et al., 2002). Damage to the IL region causes increased CRF mRNA expression in the LA (Radley, Arias, & Sawchenco, 2006). Lesion studies investigating extinction training suggest that the IL region indirectly inhibits HPA responses partially through CRF containing projections from the IL cortex to the LA (Brake, et al., 2000; Diorio, et al., 1993; Figueiredo et al., 2003; Jinks & McGregor, 1997; Spencer et al., 2005; Sullivan & Gratton, 1999, 2002).

**Stress Hyporesponsive Period**

Associative conditioning has been used in young rats to examine the developmental effects of stress on learning & memory brain structures. In a study comparing associative paradigms, Ivkovich, Paczkowski, & Stanton (1999) found that trace conditioning emerges gradually, starting around postnatal day (PND) 14 and plateaus between PND 28 and 31 due to further development of learning and memory structures. Interestingly, there is a critical period early in rat development during which the animal is shielded from the detrimental effects of elevated CORT. This period of time is called the stress-hyporesponsive period (SHRP) and it occurs from PND 2 through PND 14. During the SHRP, basal levels of CORT are greatly diminished and stress-induced increases are blunted (Sapolsky & Meaney, 1986; Walker, et al., 1991).
Following the SHRP, there is a burst of endogenous CORT and a continual rise which aids in brain development until at least the fourth week of life (Walker, et al., 1986). The period immediately following the SHRP is characterized by robust reactions to stressors. There is an inverted-U relationship of CORT treatment effects during this period of development where long-term release of CORT from subcutaneous pellets (800 ng/ml peak CORT level) and osmotic minipumps (120 ng/ml peak CORT level) resulted in impaired learning during trace eyeblink conditioning whereas intermittent subcutaneous injections of CORT resulted in facilitation of learning on the same task (Claflin, Hennessy, Jensen, 2005; Claflin et al., 2011; Kraszpulski, et al., 2012). In experiments using trace eyeblink association paradigms immediately following the SHRP, researchers found that 0.02 mg/g CORT administered over 3 days (twice daily) using subcutaneous injections (peak CORT plasma level 900 ng/ml transient) resulted in a facilitation of learning, with most of the effects sex-specific to males (Wentworth-Eidsaune, 2010). These results are consistent with literature on endogenous stressors. Endogenous elevations of CORT from stressors such as restraint stress have a similar inverted-U relationship with learning and memory. Optimal declarative memory performance occurred at moderate durations of restraint stress and stress-induced increases in glucocorticoid levels, whereas shorter or longer periods of restraint stress and stress-induced increases in glucocorticoid levels are less effective or impaired performance on these tasks (Sauro, Jorgensen, Pedlow, 2003). More research is needed to understand the effects of exogenous glucocorticoid administration on learning & memory and brain structures during this post-SHRP of development.
**CRF and development**

Developmental experiments have found that aversive stressors, such as mother separation, restraint stress, or social stress can cause CRF protein release and increased levels of CRF mRNA expression in the LA (Hsu, et al., 1998; Makino, et al., 1999; Merali, et al., 1998). Parental separation during development caused higher levels of CRF mRNA expression in CRH containing neurons in the LA of stressed animals compared to nonstressed animals while the IL region was not effected (Becker, et al., 2007; Seidel, et al., 2011). Other studies using exogenous CORT treatment have failed to find effects on the LA and IL during this post-SHRP of development (Koppensteiner, et al., 2014; Morales-Medina, et al., 2009).

Research investigating how CORT treatments and CRF mRNA expression relate to this learning & memory system has been conflicting and inconclusive for this critical period of development. More work needs to be done to characterize the learning, memory, and endocrine profile in the LA and IL following CORT treatment immediately after the SHRP. This study aims to investigate the lasting effects of 2 different exogenous CORT doses immediately following the SHRP. 2 weeks following treatment, we assessed trace fear conditioning, extinction and extinction recall, and quantified CRF mRNA in the amygdala (LA projections to CE) and mPFC (IL projections to LA) at each dosage.

**Hypotheses**

We hypothesize an inverted-U relationship of CORT effects on learning & memory behaviors as well as CRF mRNA expression. From previous trace eyeblink work showing that High-dose CORT pellets and Low-dose osmotic CORT minipumps implanted during this period of development have deleterious effects on learning and
memory while Medium-CORT injections cause a facilitation in training, we hypothesize that Medium-CORT dose injections will facilitate learning & memory performance in trace fear conditioning compared to control treatments and High-CORT injections (Wentworth-Eidsaune, 2010). We also hypothesize that CRF mRNA expression in the LA and IL will mirror these inverted-U behavioral results given the relationship between CRF and CORT.
Subjects
Timed-pregnant female Long-Evans rats were ordered from Charles River Laboratories (Raleigh, NC) around embryonic day 15. On PND 4 or 5, litters were culled to 10 pups, 5 male and 5 female whenever possible. Animals were housed with dams in a colony room containing 12” X 18” polycarbonate cages with Tek-FRESH bedding (Harlan Laboratories). Animals were maintained on a 12:12 hour light:dark cycle. Access to food and water was provided *ad libitum*. Pups were housed with dams until weaning on PND 21, at which time they were housed with same-sex littermates. The final sample size included 9 litters, providing 27 experimental males for this experiment.

Injections
On PND 15, animals were randomly assigned to one of 3 dose groups: High-CORT, Medium-CORT, and control. Injections were given twice a day for 3 days. CORT (Sigma C2505) was dissolved in sesame oil (Sigma 3547), vortexed, and maintained in a 37°C water bath 24 hours prior to administration. Subjects were treated twice daily (0900 and 1700) on PND 15, 16, and 17. The dams were removed from the home cage and the pups were moved to an experimental room where weight was recorded and doses were calculated before every treatment. The animals in the High-CORT group and the Medium-CORT groups were dosed at 0.04 mg/g or 0.02 mg/g CORT (respectively) within 0.005 ml/g oil while control animals received an equal volume by body weight of
oil vehicle alone. Treatments were introduced subcutaneously at the nape of the neck with a 26.5 gauge needle. Animals were placed in a clean cage over a heating pad following administration until they recovered and were returned to their home cage together with their mothers in the colony room.

**Behavior**

Behavioral conditioning began on PND 28. Conditioning occurred in two identical clear Plexiglass (26 cm long X 26 cm wide X 18 cm high) fear conditioning chambers inside sound attenuating boxes (Coulbourn Instruments). FreezeScan software (Clever Sys, Inc.) monitored freezing behavior and administered the learning paradigm. Freezing is characterized as a behavioral arrest elicited by a prey animal when they feel threatened. Animals received the conditioned stimulus (CS) tone (85 dB, 2 kHz, 20 seconds) and an unconditioned stimulus (US), a mild foot shock (2 seconds, 0.5 mA). The US was delivered 15 seconds after the cessation of the CS. In all paradigms, freezing was analyzed during the 15 second adaptive learning window between the tone and US, indicating the freezing response is due to the tone and predictive of the impending shock. Animals underwent 6 trials of conditioning (i.e. 6 tone shock pairings) and were then returned to their home cage. Twenty-four hours later, the animals underwent fear extinction—15 trials tone-only. The time between the trials was randomly assigned between 60-90 seconds for every trial. The fear conditioning boxes were covered with black and yellow stripes as well as polka-dots in an effort to disguise the boxes from the training day. The animals were transported to the testing room using different routes and transportation boxes, as well. Vanilla extract was applied to the bottom pan and white
Plexiglass was also placed on the above floor to further modify the boxes. Lighting in the altered testing chambers and room was dimmed and a fan ran in the background provided white noise. The animals also underwent 6 trials of extinction recall 24-hours following extinction (tone-only). This was done with the same context and surroundings as the initial fear paradigm. In between all subjects, both fear conditioning boxes were cleaned with 70% alcohol and liquinox soap.

**Brain Analysis**

24 hours after completion of behavioral testing, animals were sacrificed by CO2 inhalation and decapitation. The brains were extracted and placed on dry ice to be immediately frozen and then placed into a -80 °C freezer until sectioning. Brains were sliced at 16 microns thick with a cryostat. The IL and LA were sliced coronally and mounted with 2 sections per slide. 6 slides containing the amygdala and 6 slides from the IL region were mounted for each brain. Half of those slides were used for a thionine stain to verify our location and the other half were used for mRNA analysis. We used an oligoprobe to determine CRF mRNA expression. A 48-mer CRF mRNA oligoprobe was utilized (5’ GAC ACC GCC CAA AGC CAG GAC GAT GCA GAG CGC GGC CAG CGC GCA CTG 3’) (Falco, et al., 2009; Young, Mezey, Siegel, 1986). Procedures for hybridization followed the methodologies described by Young (1992) and used in Falco et al. (2009). Thawed, dried sections were temporarily fixed in 4% formaldehyde/PBS for 5 minutes, washed in PBS, acetylated in 0.25% acetic anhydride/1 M triethanolamine hydrochloride (pH 8.0) for 10 minutes, dehydrated in systematically increasing ethanol mixtures, delipidated in chloroform for 5 minutes, dipped in 100% ethanol, dipped in
95% ethanol, and set out to dry. 50 µl of hybridization buffer containing 50% formamide, 600 mM NaCl, 80 mM Tris–HCl (pH 7.5), 4 mM EDTA, 0.1% sodium pyrophosphate, 0.2% sodium dodecyl sulfate, 0.2 mg/mL sodium heparin, 100 mM dithiothreitol, 10% dextran sulfate, 0.01% cold polyadenylic acid, plus 1×10^6 cpm of labelled probe was then applied to each slice, and then slides were coverslipped with parafilm. Slides were incubated overnight at 37 °C. The parafilm was taken off while the slides were submerged in SSC, and slides were washed and collected in 1× SSC, rinsed in 4 changes of 1× SSC at 60 °C for 15 minutes each, and then rinsed in 2 changes of room temperature 1× SSC for 30 minutes each. Slides were washed in H₂O and 70% ethanol, and dried on the table counter. Biomax film (Eastman Kodak, New Haven, CT) was developed after exposure to a cassette containing the treated slides and 14C standards (ARC Inc., St. Louis, MO). A flatbed scanner was used to convert autoradiographic pictures of each slide into a TIFF file. Regions of interest were then sampled manually using NIH Image (Rasband, NIH), with optical density interpolated in line with the calibration curve defined by the standards. This method permitted the quantification of CRF mRNA expression changes in specific learning- & memory-related brain regions. The areas of the LA and IL were calculated according to Paxinos & Watson (1998).

**Statistics**

*Behavior.* 3 separate 2-way mixed design ANOVAs were performed to assess differences in time freezing during the trace interval between High-CORT, Medium-CORT, and Control over time in conditioning, extinction, and extinction recall. Post hoc analysis was used to assess any pairwise differences between drug groups. We also
looked for simple effects of learning over time within each CORT group, to indicate if a particular treatment caused faster change from baseline (indicating facilitated learning). Additionally, contrasts were used to analyze significant differences between the control group and both High- and Medium-Cort groups to analyze whether there was an effect of CORT, independent of dose. Other analyses were used such as one-way ANOVAs to examine differences in total time freezing and number of freezes measured per day between CORT groups.

*mRNA*. Quantitative in situ hybridization histochemistry was also conducted to measure the changes in CRF mRNA expression levels. Two 1-way between subjects ANOVAs were used to investigate CRF mRNA expression differences between CORT doses in the IL and LA. Correlations were also examined between CRF mRNA and behavioral variables.
CHAPTER THREE: RESULTS

Behavior

*Conditioning*. Learning was measured as the percentage of time spent freezing during the trace interval of each CS-US. All subjects learned the CS-US association over time; the assumption of sphericity was violated across trials so the Greenhouse-Geisser correction is used for the overall ANOVA ($F(2.6, 61.6) = 7.5, p < .05$). Simple contrast analysis revealed that rats froze less in Trial 1 compared to the later trials ($F(1, 24) = 7, p < .05$). There were no significant differences between CORT groups. Shown in Figure 1, animals reached a ceiling effect by the 3rd trial of conditioning. Interesting to note, the baseline for Trial 1 was around 70% freezing.

![Figure 1. Conditioning (percent freezing during trace interval +/- SEM)](image-url)
Extinction. Freezing was measured as the percentage of time spent freezing during the same adaptive window as conditioning. A large number of animals were sleeping from trials 8 – 15; this is different than freezing to the tone and is validated by a huge increase in lack of motion during the inter-trial intervals compared to earlier trials measured from the software, thus those trials were removed from our freezing analysis. By Trial 7, freezing levels had extinguished down to the comparable baseline levels from Trial 1 of conditioning (Figure 2); the assumption of sphericity was violated across trials so the Greenhouse-Geisser correction is used for the overall ANOVA (F(4.3, 102) = 7.6, p < .05). A polynomial contrast revealed a significant linear reduction of freezing over trials (F(1, 24) = 28.7, p < .05). There were no significant differences between CORT groups.

![Extinction Graph](image_url)

Figure 2. Extinction (percent freezing during trace interval +/- SEM)
Extinction Recall. Freezing was measured as the percentage of time spent freezing during the same adaptive window as conditioning and extinction. A large number of animals were seen sleeping during trial 6; this is different than freezing to the tone and is validated by a huge increase in lack of motion during the inter-trial interval compared to earlier trials measured from the software, thus that trial was removed from our analysis. During extinction recall, all subjects extinguished below the comparable baseline from Trial 1 of conditioning (F(4, 96) = 6.2, \( p < .05 \)). Shown in Figure 3, animals extinguished below baseline by Trial 3 but then slightly increased freezing into Trial 5. A polynomial contrast revealed this is a significant quadratic function with a reduction then slight increase in freezing over trials (F(1, 24) = 18, \( p < .05 \)). Animals in the High-CORT condition were seen sleeping during trial 5 but the proportion of total animals sleeping and lack of motion during the inter-trial interval was not large enough to completely remove this trial from our analysis. There were no significant differences between CORT groups.
A 1-way between subjects design ANOVA revealed significant effects between CORT doses and IL CRF mRNA expression (F(2, 21) = 4.84, p = .01). An LSD post-hoc test revealed that Medium-CORT caused a significant increase in CRF mRNA expression in the IL cortical region compared to both High-CORT and Control-CORT (Figure 4). There were no significant differences between CORT doses in effects on LA CRF mRNA expression (Figure 4). There were no significant correlations between CRF mRNA expression levels and behavioral variables.
Figure 4. CRF mRNA expression in IL and LA (mean μCi/g +/- SEM)
CHAPTER FOUR: DISCUSSION

No Effect of CORT on Freezing

There were no significant differences between CORT doses and learning & memory measures. In earlier trace eyeblink conditioning paradigms, identical Medium-CORT treatments resulted in a facilitation of learning, with most of the effects sex-specific to males (Wentworth-Eidsaune, 2010). The current experiment failed to extend these findings to freezing paradigms. Eyeblink paradigms, compared to fear paradigms, are able to subject the animals to many more trials during the learning process (Lennartz & Weinberger, 1992). This might make eyeblink behavior a more sensitive measure compared to other learning & memory behavioral assays.

The tone and the shock stimuli that are used during fear and eyeblink paradigms are the same with the only difference being that the shock is administered to the foot in fear conditioning and to the eyelid in eyeblink conditioning. Shown in Figures 5 and 6 in the appendix, the neural circuits underlying these two paradigms are different (Blair, et al., 2001; Fanselow & LeDoux, 1999; Kim & Thompson, 1997; LeDoux, 2000; Maren, 2001; Raymond, Lisberger, & Mauk, 1996). During eyeblink conditioning, the CR learning pathway involves mossy fiber signals from the pontine group to the purkinje cells of the cerebellar cortex and the interpositus bodies of the deep cerebellar nuclei (Tracy, et al., 2013;). The CR is generated by activation of the interpositus nucleus which projects to the red nucleus to ultimately excite efferent connections to the facial nucleus.
(Hesslow & Ivarsson, 1994; McCormick & Thompson, 1984). The US pathway is transmitted to the same purkinje and interpositus cells via efferent input from the inferior olivary nucleus (Linden & Connor, 1993; Ito, 2001; Sears & Steinmetz, 1991). The CS and US are believed to converge in the cerebellum for many reasons: lesions to this area prevent learning, neurons within the interpositus nucleus display conditioned response activity after learning, and stimulation of this region causes eyelid closure (Berthier & Moore, 1986; Berthier & Moore, 1990; Gould, Sears, & Steinmetz, 1993; McCormick, & Thompson, 1984; Steinmetz, Lavond, & Thompson, 1989). During fear conditioning, the CS and US are relayed to the LA from thalamic and cortical regions of the auditory and somatosensory systems. The CS inputs are thought to interact with the US in the dorsal subregion of the LA (Lanuza, Moncho-Bogani, Ledoux, 2008; Romanski, et al., 1993).

It could be the case that eyeblink procedures are more sensitive to learning & memory changes comd to fear paradigms. It could also be the case that CORT, in fact, has an effect in the eyeblink learning & memory circuitry but does not directly affect fear conditioning pathways.

**High Baseline and Conditioning Ceiling Effect**

Animals exhibited a high level of anxiety in response to the initial tone, exhibiting baseline freezing at 70% before a shock was administered. This finding is perplexing, given the extensive handling of subjects prior to testing; more work needs to be done to uncover the cause behind this effect.

A high baseline gave less room to see conditioning effects. Animals were fully conditioned to the tone by the second trial and reached an upper limit of freezing.
behavior. A follow-up experiment to optimize our learning paradigm sought to prevent this ceiling effect. Studies have shown that the trace interval can be augmented to make the paradigm more challenging (Curzon, Rustay, & Browman, 2009). This was attempted by incrementally increasing the trace interval from 15s, 30s, 45s, and 60s. A one-way ANOVA between trace intervals and across trials was not significant (F(10, 30) = .820, \( p = .613 \)). Graphically represented in Figure 7 in the appendix, lengthening the trace interval had no influence on freezing nor the ceiling effect. Subjects in the trace optimization experiment had a baseline freezing rate of 70% and reached an upper limit of freezing by the second trial. Future experiments could add in a distractor during the trace interval (such as a flashing light) to tax attentional resources and make the trace paradigm harder (Han et al., 2003). One possibility for the high baseline is that all handling and husbandry of the rats was performed by males. A recent article has suggested that rats handled by male husbandry and experimenters have a heightened stress response which manifests as a high baseline in behavioral fear testing (Sorge, et al., 2014). It is also possible that the 85dB tone used in this study was excessively loud and cause a heightened response in animals of this age. Though previous eyeblink work during the same developmental period used a 90 decibel tone (5dB higher than the present study), a tone of this intensity could have been a factor in our heightened baseline to tone 1.

**CORT Effects on CRF mRNA in the IL and LA**

In line with our Yerkes-Dodson relationship of glucocorticoid effects, our Medium-CORT dose increased CRF mRNA expression in the IL cortex while the control
group and High-CORT group did not cause significant changes. The mechanism causing CRF mRNA expression effects from CORT doses of 0.02 mg/g but not 0.04 mg/g CORT is unknown. To our knowledge, this is the first study to show that chronic CORT treatments cause lasting effects in CRF mRNA expression in the IL cortex when administered during this post-SHRP of development in rats. These results could suggest that elevated CRF mRNA expression might be a cause for the reduction in IL dendrites shown in previous experiments administering 0.025 mg/g subcutaneous CORT (Cerqueira, et al., 2007). While the IL cortical region is involved with the extinction of learned associations, future studies interested in glucocorticoid effects on CRF and prefrontal functioning could also examine the prelimbic cortical area which is found just dorsal to the IL and is critical in the acquisition of fear conditioning (Sierra-Mercado, Padilla-Coreano, & Quirk, 2011).

It is also interesting that there is a regional dissociation in these CORT effects as the LA did not show changes in CRF mRNA expression in the present experiment. As mentioned, endogenous stressors during this developmental period, such as parental separation, cause higher levels of CRF mRNA expression in CRH containing neurons in the LA of stressed animals compared to nonstressed while the IL region has not been found to be effected by these stressors (Becker, et al., 2007; Seidel, et al., 2011). This gives new insight into the differences between endogenous stress mechanisms versus direct glucocorticoid administration effects on the LA and IL.
Conclusions

In line with our hypothesis, glucocorticoids do have an inverted-U relationship with CRF mRNA expression. Subcutaneous CORT treatments of 0.02 mg/g administered for 3 days, twice daily immediately following the SHRP caused heightened CRF mRNA expression in the IL cortex 2 weeks following treatment compared to control groups and a dose of 0.04 mg/g CORT. However, even though identical procedures using 0.02 mg/g injected CORT had effects on trace eyeblink conditioning in previous studies, we were unable to extend these results to trace fear conditioning. It is possible that the reason we see a difference in CORT effects on trace eyeblink versus fear paradigms is the differences in underlying circuitry of the memory systems. It could be the case that CORT has an effect on the CS-US convergence seen in the interpositus nucleus of the cerebellum during eyeblink conditioning while it does not have an effect on the lateral amygdala, where the CS and US converge in fear conditioning. In this study, LA CRF mRNA expression was not effect by glucocorticoid treatments while the IL cortex showed a 2-fold increase in CRF mRNA compared to control and high-dose treatment. Our findings suggest that the cause of endogenous and exogenous stress effects on the IL cortex (such as CRF mRNA expression changes from CORT injections) might be a result of circulating glucocorticoids while the causes of stress effects on the LA in other studies come from another mechanism. Future studies interested in glucocorticoid effects on CRF and prefrontal functioning could examine the prelimbic cortical area which is found just dorsal to the IL and is critical in the acquisition of fear conditioning.
REFERENCES


the forebrain depending on type of stressor. *European Journal of Neuroscience, 18*, 2357-2364.


Moraes-Medina, J.C., Sanchez, F., Flores, G., Dumont, Y., & Quirion, R. (2009). Morphological reorganization after repeated corticosterone administration in the...


Vallandingham, Z. (2012). Effects of elevated glucocorticoid levels on dentate gyrus development. (Electronic Thesis or Dissertation; Wright State University).


Wentworth-Eidsaune, C. (2010). *Short-Term Administration of Corticosterone has Lasting Effects on Learning in Young Rats.* (Electronic Thesis or Dissertation; Wright State University).


Subcutaneous pellets and osmotic minipumps resulted in impaired learning during trace eyeblink conditioning whereas subcutaneous injection of CORT resulted in facilitation of learning on the same task (Claflin et al., 2005, 2011). One of the possible mechanisms for these CORT-induced cognitive effects may be changes to hippocampal development, specifically neurogenesis. Researchers examined potential differences in hippocampal neurogenesis for rats subjected to elevated CORT levels as in the aforementioned behavioral studies. In these neurogenesis experiments, exogenous administration of CORT was administered via subcutaneous pellets. CORT-treated males showed significantly less neurogenesis compared to control animals (Vallandingham, 2012). CORT-treated males also showed significantly less neurogenesis than the CORT treated females. Furthermore, males of the control group showed significantly more neurogenesis than the females. Given the substantial evidence of CORT effects on hippocampal development immediately following the SHRP, we hypothesize that we will see effects of our CORT treatments on hippocampal CRF mRNA expression in the dentate gyrus. Specific fields in the hippocampus will be investigated in future analyses. A 2 week film exposure was not enough to image CRF mRNA levels in the hippocampus; our next film exposure will run for 2 months to ensure our ability to image and analyze CRF mRNA expression in the hippocampus.
Figure 5. Underlying circuitry relevant for fear conditioning and extinction in this experiment
Figure 6. Underlying circuitry for eyeblink conditioning
Figure 7. Trace optimization conditioning graph (percent freezing during trace interval +/- SEM)
BIOGRAPHY

Kevin Schmidt graduated from Kings High School, King Mills, Ohio, in 2007. He received his Bachelor of Science from Wright State University in 2011. He was employed by the Air Force Research Laboratory and was awarded the Science Mathematics and Research for Transformation scholarship for service to pursue his Master of Arts in Psychology at George Mason University in 2012 to be gainfully employed by the Air Force Research Laboratory upon completion.