QUANTIFYING PLASMA CORTICOSTERONE LEVELS

FOLLOWING A CAREGIVING PARADIGM

OF EARLY-LIFE ADVERSITY

by

Lauren Reich

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 Biological Sciences with Distinction

Spring 2019

QUANTIFYING PLASMA CORTICOSTERONE LEVELS

FOLLOWING A CAREGIVING PARADIGM

OF EARLY-LIFE ADVERSITY

by

Lauren Reich

Approved: _____
Tania L. Roth, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved: _____
Carlton R. Cooper, Ph.D.
Committee member from the Department of Biological Sciences

Approved: _____
Jeffery Rosen, Ph.D.
Committee member from the Board of Senior Thesis Readers

Approved: _____
Michael Chajes, Ph.D.
Chair of the University Committee on Student and Faculty Honors

ACKNOWLEDGMENTS

I would first like to thank Dr. Tania Roth for being such an incredible mentor. As I have grown in the lab, she has always supported and cared for me and my endeavors. I have watched her persevere through too many obstacles since I joined the lab in the fall of 2016, and since then I have admired her unyielding strength and resilience. Through these hardships, Dr. Roth has never failed to be patient and kind to her students. I am truly thankful to have spent the last two and a half years under her incredible mentorship and know that she will always support me and my goals.

I next want to recognize Dr. Tiffany Doherty for her endless guidance and love. She has taught me nearly everything I know and has trusted me even when I have made stupid mistakes. She has always believed in me and has genuinely molded me both into the researcher and person that I am today. Tiffany is not only an amazing mentor, but an even better friend. I am glad to be graduating at the same time as her because I cannot imagine her not being next to me cheering me on.

I would also like to thank my fellow undergraduate Hannah Duffy. We spent many hours together running our paradigm and performing extractions. Throughout this project, she kept me sane and on-track. I am so glad to have gotten to know her through my time in the lab and to have gained a new friend.

All of my work in the lab would not have been possible if it were not for Johanna Chajes. In the fall of 2016, she overheard me talking about my interest in epigenetics and without knowing me, approached me and encouraged me to apply to

join the lab. Without her, I am not sure the form my path would have taken. For this, I cannot thank her enough.

I would like to mention Natalia Phillips and other members of the Roth lab, both graduate and undergraduate. Thank you so much for an amazing few years filled with laughter and happiness. I would like to also thank Dr. Carlton R. Cooper and Dr. Jeff Rosen for serving on my thesis committee and for their guidance throughout this process. I would like to also thank my parents, who try their best to understand my work, but always support me regardless.

TABLE OF CONTENTS

LIST OF FIGURES

ABSTRACT

Stress is a common stimulus for both animals and humans. To process stressful stimuli, the hypothalamus-pituitary-adrenal (HPA) axis is crucial in the physiological cascade that produces glucocorticoid stress hormones: cortisol in humans and corticosterone in rodents. Glucocorticoids are essential for proper development, circadian rhythm, and behavior; however, overexpression of these hormones can result in damaging physiological and behavioral changes, often through the modification of transcription and the epigenome. Previous work in our lab has determined behavioral and epigenetic consequences in rodents exposed to our well-established seven-day caregiving behavioral paradigm of early-life adversity. External research has indicated a stress hyporesponsive period in infant rodents, where even in the presence of aversive and stressful stimuli, a pup's HPA axis will not be activated. Here we test whether our aversive caregiving conditions evoke corticosterone production, as corticosterone could be involved in our previously discovered behavioral and epigenetic consequences of the caregiving paradigm. Results indicated no significant change in corticosterone levels based on treatment group or sex. These data are consistent with other work showing the lack of a corticosterone response to stressful and aversive stimuli during the stress hyporesponsive period, and suggests that our behavioral paradigm induces epigenetic, behavioral, and neurological changes without the influence of increased corticosterone.

Chapter 1

INTRODUCTION

1.1   Response to external stressors

1.1.1   Historical context

In the field of psychology, there has historically been a debate of "Nature vs Nurture". Proponents of the nature camp argue that human behavior is dictated solely by DNA sequence and is determined since birth. On the contrary, proponents of the nurture camp believe the environment is the primary reason to describe how individuals act and behave. Evidence that emerged in the early 2000s has helped us see that a blend of the two underlies behavior (Abrahamson, Baker, & Caspi 2002; Belsky et al., 2003). Further, our genes are up- and down-regulated based on changes in the environment (Moore, 2015), as the human body is a complex system that must adapt and exist in less-than stagnant situations. This change of gene expression is modulated by several pathways and varies on the type of stimulus imposed. Often, these transcriptional changes are dictated by epigenetics, where the genome is modified to have a change in gene expression to better suit the cell's needs. In regard to tissue-wide modifications, these can influence physical health, mental health, and behavior (Ehrlich 2002; Casavant, 2019; Hamilton et al., 2019). These modifications vary in genomic location, chemical modification, and consequence, showing the remarkable adaptability of a living system.

1.1.2 Stress and biological outcomes

A stressful event is known affect an individual's wellbeing. In particular, early-life stressful experiences are widely known to contribute to negative outcomes in adulthood, often by increasing the likelihood of disease and/or risk-taking behavior (Nettis, Pariante, & Mondelli, 2019; Nieves et al., 2019; Lähdepuro et al., 2019). Early-life stress has also been linked to a higher likelihood of disease-related death in a dose-dependent association; a higher frequency of early-life hinted at a greater risk for mortal diseases such as cancer and cardiovascular disease (Russ et al., 2012).

Often, the caretaker is a common donor of early-life trauma due to the heightened dependence of a child to an adult. It is estimated that one out of every eight children experiences maltreatment from their caregiver (Wildeman et al., 2014). In altricial animals, offspring are heavily dependent on the caretaker in the early stages of life. It is known that these early interactions between caretaker and infant are crucial for neurological and behavioral development (Suomi, Harlow, & McKinney, 1972; Heim et al., 2009; Chambers, 2017). A fundamental study focusing on negative experiences related to caretaking environment is the Adverse Childhood Experiences (ACE) study. Using a questionnaire asking adult participants about their adverse experiences in their childhood household, researchers were able to link the frequency of early-life adverse experiences to mental, social, and physical deficits in adulthood. These deficits ranged from obesity, mood and anxiety disorders such as depression and posttraumatic stress disorder, and drug use (Felitti et al., 1998). Studies continue to provide support for early-life adversity as a predictor of negative adult outcomes (Anda et al., 2007; Brown et al., 2010). Due to the prevalence of these associations and the need to understand how these deficits manifest, it is crucial to better

understand how the body responds to developmental stressors, as this could allow us to create novel treatment strategies to combat these devastating outcomes.

1.2 The role of the HPA axis in stress response

Multiple regions of the nervous system are profoundly affected by developmental stressors (de Kloet et al., 1998; Doherty, Forster, & Roth, 2017). Of particular interest for this thesis study is the hypothalamus-pituitary-adrenal (HPA) axis; a stress-activated cascade that results in glucocorticoid release (Ulrich-Lai & Herman, 2009). When an organism is under stress, the hypothalamus is the first brain region stimulated. This tissue releases corticotrophin releasing hormone (CRH) and vasopressin into the medial eminence, where they can travel to various regions of the body. Together, the two hormones act on the anterior pituitary gland to stimulate the secretion of stored adrenocorticotropic hormone (ACTH). ACTH in turn is released into the bloodstream to stimulate the cortex of the adrenal glands. This adrenal activation initiates the synthesis and release of glucocorticoid hormones: cortisol in humans and corticosterone in most animal systems (Ulrich-Lai & Herman, 2009). When entering the bloodstream, these hormones are generally bound by glucocorticoid-binding globulin, which may facilitate the hormone's entry into the cell (del Mar Romero et al., 2013). Once inside, the glucocorticoid can bind to an intracellular receptor and create a receptor-ligand complex that can interact with the promoter regions of many genes, thereby ultimately regulating transcription. These effects can take minutes to hours to unfold, however generally produce an effect on metabolic, immune, and cognitive function, generally acting catabolically (Sapolsky, Romero, & Munck, 2000; Sapolsky & Meaney, 1986). However, a receptor-ligand

complex working alone tends to repress transcription, while a dimer of two receptor-ligands tends to activate transcription (Gray, et al., 2017).

Two receptors exist for these hormones: mineralocorticoid receptors and glucocorticoid receptors. The first is restricted predominantly to the hippocampus, while glucocorticoid receptors have a more widespread distribution in the brain (Reul & de Kloet, 1985). Although the mineralocorticoid receptor has a higher affinity for these stress hormones than the glucocorticoid receptor, the latter is thought to be the true culprit of receiving and creating consequences of the stress response (Pariante & Lightman, 2008). Due to the lower affinity of glucocorticoids to the glucocorticoid receptor, the body will only undergo a stress response if a flood of glucocorticoids enters the blood stream (Pariante & Lightman, 2008). If mineralocorticoid receptors were the body's detection system for the stress response, the system would likely be in a hyper-responsive state due to its high affinity for glucocorticoids. For this reason, it is widely understood that the glucocorticoid receptor is responsible for the somatic stress response (Pariante & Lightman, 2008).

The HPA axis operates on a negative-feedback loop, as increased glucocorticoids bind to receptors located in both the pituitary and hypothalamus to stop the potentiation of the stress-induced response (Ulrich-Lai & Herman, 2009). However, in situations of prolonged stress, the stressful stimulus can override the negative feedback loop and continue glucocorticoid production, thereby increasing basal levels, as seen in those who have underwent trauma (Koss & Gunnar, 2018). Not all incidences of HPA activation are negative however, as glucocorticoids are necessary for brain maturation in regard to axon and dendrite remodeling, as well as glucose utilization (Meyer, 1983). Not to mention, these glucocorticoids are essential

for the maintenance of circadian rhythms (Gray et al., 2017). Therefore, the HPA axis is crucial for proper physiological and neurological function.

1.3 Relationship between glucocorticoids and epigenetic modifications

1.3.1 Epigenetics: an overview

The link between developmental stress and negative outcomes in adulthood is often thought to be the work of epigenetic modifications in the brain (Vaiserman & Koliada, 2017). When a cell receives a chemical stimulus, temporary modifications in transcription can be induced via a second messenger system. However, for genes that need to be up- or down-regulated for a set amount of time, the cell primarily uses epigenetic modifications to accomplish this (Kanherkar, Bhatia-Dey, & Csoka, 2014). Genes that are not always constitutively active or inactive such as those involved in development, specialization, or homeostasis, are often epigenetically regulated (Mortada & Mortada, 2018; Matilainen, Quirós, & Auwerx, 2017). There are two common locations for these modifications: on the DNA itself, and on the amino acid tails of histone proteins, of which DNA wraps around (Moore, 2015). There are many different kinds of modifications that can be added to these locations, such as methylation, acetylation, and ubiquitination to name a few, however much is still unknown about their total effects on transcription (Moore, 2015). By investigating specific genes, experiments, and/or compounds, researchers are able to better understand how these modifications influence a living system.

1.3.2 Glucocorticoid-mediated epigenetic modifications

Corticosterone, a glucocorticoid, induces epigenetic modifications (Ewald et al., 2014). As glucocorticoids are known to induce a wide array of changes to

metabolism, development, and behavior, it is clear there must be many mechanisms by which these compounds behave. Ewald et al. (2014) focused on the ingestion of glucocorticoids and its significant increase in the methylation of a particular gene known to be sensitive to stress. Although a stressful stimulus was not given, the influx of stress hormone acted as a physiological stressor. This identifies a clear link between glucocorticoids and epigenetic modification, however much is still unknown about the full epigenetic capabilities of these stress hormones. Glucocorticoids also can act as a negative feedback loop to the HPA axis when they bind to receptors in the brain (Ulrich-Lai & Herman, 2009). As the brain is extremely intricate and influences many downstream processes, there is question as to what these bindings effect on a full-body level. Interestingly, the bound glucocorticoid receptor is known to have complex interactions with the genome that are still not understood (Hunter et al., 2014).

1.3.3   Manipulation of HPA axis activation

Regulation of the glucocorticoid response can also be mediated by regulating the genes critical for glucocorticoid detection and regulation of the HPA axis. Following a stressful event in early life, many studies have found significant changes on both the epigenetic modification and transcriptional levels of the gene coding for the glucocorticoid receptor (Weaver et al., 2004; McGowan et al., 2009; Perroud et al., 2011). By modifying the incidence of receptor formation, the cell has the ability to prevent or incite the HPA response, influencing many downstream processes that are associated with this protein. Studies show a link between increased DNA methylation, the decrease in glucocorticoid receptor transcription levels, and a history of adverse experiences in early-life (Weaver et al., 2004; McGowan et al., 2009; Perroud et al., 2011). As DNA methylation is known to suppress the transcription of a gene,

increased methylation of the glucocorticoid receptor gene suggests a decrease in the number of translated glucocorticoid receptors. This can therefore lead to a change in cellular response, as it decreases the number of receptors available for stress hormone detection. Another study highlights a microRNA localized in the brain that has been shown to decrease expression of the glucocorticoid receptor gene (Vreugdenhil et al., 2009). As seen here, the cell has multiple mechanisms to regulate the amount of glucocorticoid receptor being produced, thereby modifying the stress response which would have behavioral consequences.

1.3.4   Behavioral and developmental outcomes

The consequences of these modifications can potentially result in the change of behavior. This has been demonstrated in a study by McGowan et al., (2009), where suicide patients with a history of child abuse were found to have decreased gene expression of the hippocampal glucocorticoid receptor gene. As described earlier, multiple studies have found increased methylation of the gene responsible for glucocorticoid receptor formation in subjects exposed to early-life adversity (Weaver et al., 2004; McGowan et al., 2009; Perroud et al., 2011). Studies have also shown that a selective knockdown of glucocorticoid receptor expression in rodents is associated with increased HPA axis activity under both normal and stressful conditions, showing a hyperactive behavioral response regardless if a stressor is present (Ridder et al., 2005). Furthermore, mice with lower glucocorticoid receptor concentration were found to exhibit depressive-like behaviors (Ridder et al., 2005). This creates a connection between early-life adversity/stress, the physiological deficit of glucocorticoid receptors, and anxious or depressive tendencies.

De Kloet et al. (1998) further connects the influence of the HPA axis on the brain, showing that disturbances in mood, cognition, in behavior often correlate with abnormal levels of glucocorticoids themselves. In fact, the study shows that many patients who suffer from major depression have hyperactive HPA axis function, resulting in increased levels of glucocorticoids. Researchers believe the heightened HPA axis response is related to reduced feedback inhibition by endogenous glucocorticoids due to a lack of functioning glucocorticoid receptor (Pariante & Lightman, 2008). On the other hand, glucocorticoid receptor overexpression is associated with a lesser HPA axis response to acute stress (Reichardt et al., 2000). Both this increased reactivity and stress numbness can be detrimental in certain stressful situations. Although complex, a balance is critical for proper neurological function and survival.

Multiple human studies showcase the physiological effect of increased cortisol production. Researchers have found a link between elevated cortisol and reduced volume in the left hippocampus (de Kloet et al., 1998). Studies have found cortisol to influence the epigenome of specific age-related genes, which therefore cause a shrinkage of the brain region (Davis et al., 2017). Further studies on cortisol increase have also examined a decrease in the brain-derived neurotrophic factor gene, which is crucial for proper brain development (de Kloet et al., 1998; Smith et al., 1995; Li et al., 2019). This has also been implicated with other factors, such as the nerve growth factor, basal fibroblast growth factor, and transforming growth factor specifically in the hippocampus (de Kloet et al., 1998). It is clear that heightened cortisol has connections to brain and gene development, thereby influencing cognitive processing and future growth.

Early-life experiences are known to leave a long-term influence on the activity of the HPA axis. Rodents raised in varying nurturing conditions have a heightened response to further stress later in life. Champagne et al. (2003) shows the difference between rodent offspring exposed to high-frequency licking/nursing mothers and low nurturing mothers. Offspring with mothers who exhibited a low frequency of nurturing behaviors displayed a higher corticosterone response when in acute stress compared to their high nurtured peers. The low-nurturing dams may have acted as an early-life stressor or aversive stimulus, unable to provide for the pups when needed, thereby resulting in this HPA sensitivity. In humans, this heightened glucocorticoid response was also found in adult women who had undergone a serious trauma in childhood when later given a stressful stimulus (Heim et al., 2000). This shows the powerful influence of early-life experiences and their ability to alter stress reactivity later in life.

1.4 Stress hyporesponsive period

The influence of a caretaker is critical in the development of an infant (Greenberg, Speltz, & Deklyen, 1993). As humans can care for a crying child by nursing or rocking them, rodents often care for their young by licking and/or grooming them. While this promotes a bond and stimulates neuronal growth due to positive stimuli, this behavior also protects them against heightened corticosterone responses due to early-life stress (Weaver et al., 2004; Murgatroyd et al., 2015). Studies have demonstrated that the infant rodent is relatively unresponsive to stressors during the first few weeks of life if the mother is presenting nurturing behaviors (Loman & Gunnar, 2010). Specifically, immediately after birth, pups' basal corticosterone levels begin to sharply decrease and remain at extremely low levels until approximately postnatal day 14 (Levine, 1994). Although following a different timeline, this same

phenomenon occurs in non-human primates and humans (Wiener et al., 1990; Loman & Gunnar, 2010).

This stress hyporesponsive period is suggested to protect the animal from major transcriptional changes while in a critical neurodevelopmental period (Lupien et al., 2009). Glucocorticoids have been found to be involved in the inhibition of cell division (Sapolsky & Meaney, 1986). In the developing rodent, this could create devastating consequences, as large spikes of growth are present in early-life. A delicate balance of glucocorticoids is needed, as both too high and too low levels of these hormones can interfere with development (Levine, 1994). Too high of levels can interfere with central nervous system development, however a low amount is essential; glucocorticoids are necessary for arousal, as it is involved with epinephrine secretion (Sapolsky & Meaney, 1986).

During the stress hyporesponsive period, the caretaker protects the subject from having a physiological response to stress. However, if the maternal bond is not formed, there is a lack of ability for the infant rat to gain this protective period. This can result in spikes in corticosterone response for the infant, even when subjected to low stress (Champagne et al., 2003). Without this nurturing behavior, the subject is more sensitive to stressors in the future, often creating a hyperactive somatic response (Loman & Gunnar, 2010). Human infants with an insecure attachment to their parental figure have difficulty moderating their cortisol spikes even when in the presence of a parent (Hertsgaard et al., 1995). This further shows the influence of a parental figure on an infant's feelings of security, and its suggestions to irregular neurological development.

1.5 Maternal maltreatment as a form of early-life adversity

As mentioned previously, the interaction between mother and infant is known to have a large effect on the infant's development (Greenberg, Speltz, & Deklyen, 1993) In our lab, we utilize a caregiving paradigm of early-life adversity to mimic maltreatment. By manipulating a rodent dam's access to nesting resources in a novel environment while caring for infant pups, we can promote either negative or positive caregiving behaviors. Our well-established caregiving model of early-life adversity has been utilized for many experiments in our lab (Roth et al, 2009; Blaze et al., 2013; Doherty et al., 2017). Novel environments are known stressors for rodents, and by preventing habituation to this novel enclosure, we can produce a stressed or unstressed response. Furthermore, to properly care for young, rodents often build nests out of available materials, which in our case, is bedding material. By giving limited bedding, we can promote erratic caregiving behaviors. By manipulating these variables, we are able to provide an experimental setting to examine the effects of maternal maltreatment.

Multiple studies in our lab (Keller, Doherty, & Roth 2018; Doherty, et. al., 2017; Blaze & Roth 2017) have analyzed video and audio (both audible and ultrasonic) recordings taken during behavioral manipulations to determine if treatment groups are indeed receiving different caregiving behavior. For example, Keller, Doherty, & Roth (2018) demonstrated a significant difference in adverse and nurturing behaviors from dams assigned to the maltreatment group. This indicates that infants in the maltreated condition receive significantly more adverse and less nurturing behavior than their normal maternal care and cross-foster care counterparts (Figure 1).
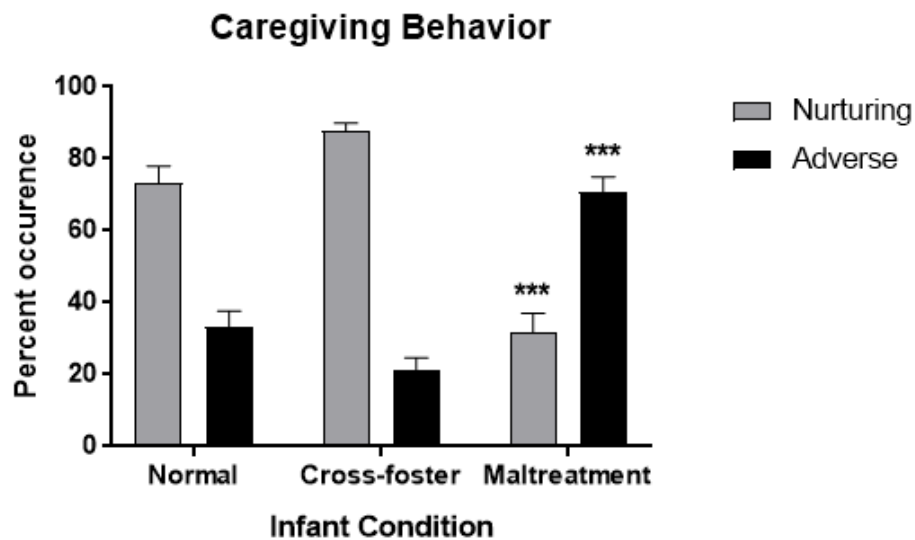
Caregiving Behavior

Figure 1. Data recreated from Keller, Doherty, & Roth 2018 shows the distribution of
adverse and nurturing behaviors during infant manipulations. The
maltreatment group has significantly less nurturing and more adverse
behaviors relative to subjects within the normal maternal care and cross-
foster conditions. n=5 dams; error bars represent SEM; *** denotes
$p<0.0001$.

Regarding pup vocalizations, Blaze & Roth (2017) measured vocalization

frequency to determine if there was a difference between treatment conditions. As

human infants cry out for help, rodents do the same, therefore giving researchers an

ability to document a difference in experience and the caregiving behavior they

receive (Portfors, 2007). Blaze & Roth concluded a significant increase in audible

vocalizations in the maltreatment group when compared to the normal maternal care

group. A marginal increase was measured in comparison to the cross-foster group

(Figure 2A). For ultrasonic vocalizations, significant increase was found in the

maltreatment condition when compared to both normal maternal care and cross-foster

care subjects (Figure 2B). This shows that pups in the maltreatment condition have

increased vocalizations, thereby showing they receive more adverse care.

Audible Vocalizations

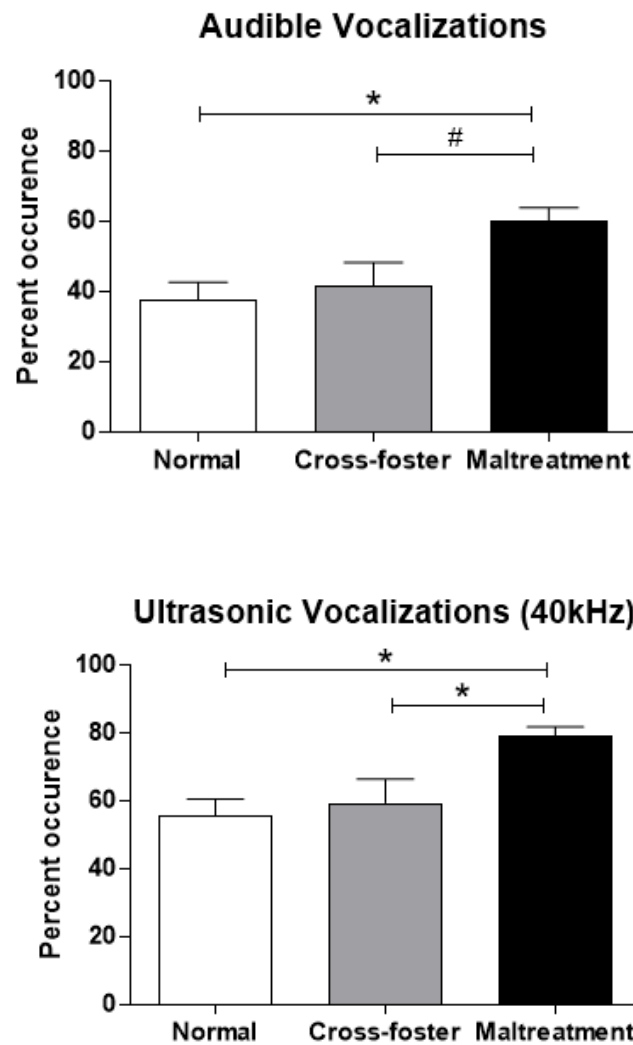**Ultrasonic Vocalizations (40kHz)**

Figure 2. Data recreated from Blaze & Roth 2017. Shows the difference in pup
vocalization across treatment groups. Pups exhibit significantly more (A)
audible and (B) ultrasonic vocalizations in the maltreatment condition
than their counterparts in the normal maternal care condition. n=7 litters;
error bars represent SEM; * denotes $p<0.05$; # denotes $p=0.079$.

In regard to the infant pups, previous experiments have shown significant changes in brain development in multiple regions (Roth et al., 2009; Blaze & Roth 2017; Doherty, Forster, & Roth, 2016). These studies cite a variety of neural changes, such as alterations in gene expression via the manipulation of epigenetic markers. A conclusion of particular impact is from Roth et al. (2009), that discovered methylation of the brain-derived neurotrophic factor (*Bdnf*) gene following the caregiving paradigm. Crucial for proper brain development, the gene codes for a protein of the same name. This protein is responsible for promoting neuron growth, maturation, maintenance, and synaptic function (Charbonneau & Healy, 2005). The methylation of this gene provides insight onto how adverse caregiving behavior influences the brain. Studies from our lab have emphasized the behavioral outcomes of adult subjects that were exposed to our caregiving paradigm as infants (Doherty et al., 2017). From Doherty et al., (2007), results concluded an alteration in adult rodents' cognition, memory, and depressive-like behaviors following the adverse caregiving paradigm. From this, it is clear that our caregiving paradigm has consequences that span the lifetime. Further, our work has showed some causality between the epigenetic modifications and behavioral outcomes (Keller, Doherty, & Roth, 2018).

1.6 The current study

Our caregiving paradigm has shown the effects of early-life maltreatment on the development of the rodent brain, as well as phenotypic outcomes later in life. Although from a conceptual and psychological point of view we utilize a behavioral model of stress, biological analysis on stress hormones has not been completed. As corticosterone has been found to be an epigenetic modifier, we are often asked as a laboratory about corticosterone levels following our paradigm. This is of particular

interest as the HPA axis is known to affect the epigenome and modify the transcription of various genes, such as our closely examined *Bdnf* (de Kloet et al., 1998; Smith et al., 1995; Li et al., 2019; Davis et al., 2017).

Thus, here we examine whether pups experience a corticosterone response following exposure to our caregiving paradigm, particularly the maltreatment condition. Based on the stress hyporesponsive period literature discussed earlier, we predicted we would not see a significant corticosterone response.

Chapter 2

METHODS

2.1 Subject generation

Long-Evans rats were bred in house with six total experimental litters produced from three dams. Breeder males and dams were only paired once to promote genetic diversity between our litters. Dams were bred initially with one litter produced before being used in experiments to control possible confounding variables associated with being a first-time mother. All animals were housed in 18 x 9 x 8in polypropylene cages, given ad libitum access to food and water, and maintained on a 12-hour light/dark cycle (lights on at 7:00 am). Day of birth was marked as postnatal day (PN) 0. On PN1, litters were culled to 12 pups when possible.

2.2 Caregiving behavior paradigm

The scarcity-adversity model of low nesting resources has been previously utilized by our lab (Roth, et al., 2009; Blaze, et al., 2013). Using a within litter design, pups were randomly divided into three caregiving groups: normal maternal care, cross-foster care, and maltreatment. For 30 minutes per day for seven days of early life (PN1 – PN7 or PN2 – PN8), pups underwent varying amounts of maternal caregiving or stress based on treatment condition. In the normal maternal care group, pups from the experimental group were weighed and immediately returned to their biological dam. In the cross-foster care group, pups were placed with an age- and diet-matched dam in a chamber (45.5 x 30.5 x 45cm Plexiglass chamber) with abundant nesting resources. Immediately prior to experimentation, the dam was given one hour to adequately habituate to her environment. Both the ample nesting resources and the habituation time are known to elicit nurturing maternal behaviors (Blaze, et al., 2013).

This is contrary to the maltreatment group, where pups and an unhabituated dam (also age- and diet-matched to the biological dam) were placed in a similar chamber with scarce nesting resources to promote erratic and aversive caregiving behavior (Blaze, et al., 2013). Following the 30-minute exposure, pups were placed back with their biological mother. Manipulations were performed at varying hours each manipulation day (during the light cycle) as to reduce any expectancy and to continue to promote a stressful environment for the maltreatment condition.

2.3 Biochemical analyses

On PN8 or PN9 (dependent on when manipulations began) after behavioral manipulations were completed, subjects were removed from the home cage, euthanized, and trunk blood was collected with 50μL of EDTA in order to prevent clotting. Collection was consistently performed in the afternoon to prevent time of day from being a confounding variable, as corticosterone levels follow a circadian rhythm (Halberg, 1969; Koss and Gunnar, 2018). Blood samples were spun down at 18,213 rcf for 10 minutes to segregate plasma from blood cells. A range of 20 to 600 μL of plasma were collected due to errors during blood collection. Once isolated, plasma samples were kept at -80 °C until further use. Corticosterone concentration was calculated by first generating a standard curve with provided standards (Corticosterone Enzyme Immunoassay Kit, Arbor Assays, Ann Arbor, MI). From this standard curve, unknown concentrations could then be determined for analysis. Duplicates of both standards and unknown samples were used to provide a more accurate reading.

2.4 Statistical analyses

Subject data was segregated by both treatment condition and sex. Two initial outliers due to extrapolation from the generated standard curve were excluded (MyAssays). Two remaining outliers within treatment condition and sex were also excluded (GraphPad Prism). If after the exclusion of outliers, a litter contained more than one pup in a particular treatment group and were of the same sex, their corticosterone values were averaged. This was done to avoid oversampling from a litter. A two-way ANOVA was used to decipher significance in sex and/or infant treatment condition.

Chapter 3

RESULTS

3.1 Corticosterone

For the current study, if a litter contained more than one pup of the same sex per treatment group, their corticosterone results were averaged to avoid over-sampling from within a litter (Holson & Pearce, 1992). A two-way ANOVA was used to assess corticosterone levels across treatments and sexes. As seen in Figure 3, there was no significance between treatment groups $[F_{(2,27)}=0.65, p=0.53]$ or sexes $[F_{(1,27)}=0.14, p=0.71]$. In addition, there was no interaction between variables $[F_{(2,27)}=0.76, p=0.48]$.
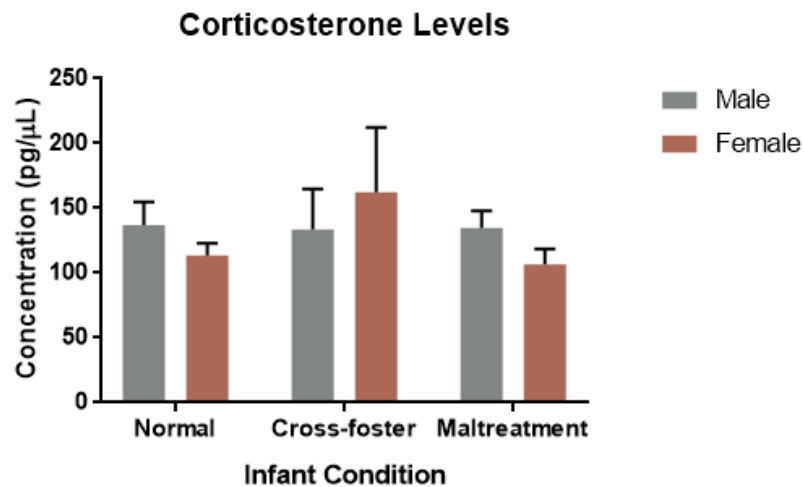

Figure 3. Depicts the concentration of plasma corticosterone across treatment condition. No significance was found between treatment groups. n = 5-6 pups/group; error bars represent SEM.

3.2 Extraction day variability

Due to varying birthing times between the three treatment groups, three of the six total experimental litters generated were sacrificed on post-natal day 9 instead of the traditional post-natal day 8. To determine if this was a confounding variable, a two-way ANOVA was performed between infant condition and day of extraction. Values for both sexes were combined and averaged as there was no sex effect in the previous analysis. There was no main effect of treatment condition [$F_{(2,27)}=0.58$, $p=0.57$] or day of extraction [$F_{(1,27)}=0.36$, $p=0.55$]. In addition, there was no interaction between these two variables [$F_{(2,27)}=2.29$, $p=0.12$]. Overall, this provides insight that the 24-hour difference between extraction times had no measurable consequence.

Chapter 4

DISCUSSION

4.1 Caregiving paradigm successfully elicits treatment-dependent maternal behavior

The aim of this study was to determine if our caregiving paradigm was activating the HPA axis, the body's primary response to a stressful stimulus. To do this, we measured pup plasma corticosterone levels following our seven-day caregiving paradigm. By measuring this stress hormone following our behavioral manipulations, we were able to assess the impact of our paradigm on HPA axis activity.

To definitively make conclusions about the consequences of our paradigm, we must first be certain that it truly manipulates caregiving behavior and that pups are responding differently to the treatments. Dams allocated to the maltreatment condition are placed in a completely novel environment with little to no bedding during the behavioral session. By manipulating these parameters, we promote erratic maternal behavior. As pups are subjected to these adverse behaviors, they vocalize, a measure suggesting their distress (Portfors, 2007). Due to time constraints, we were unable to analyze caregiving or vocalization behavior for the cohorts of animals used in this study. However based on previous observations summarized in Figures 1 and 2, we are confident that our paradigm provides adversity in the context of caregiving.

4.2 The stress hyporesponsive period protects our subjects from our stress-inducing behavioral paradigm

As a paradigm with a length of only 30 minutes per day for the first seven days of life, our paradigm could be considered only a mild stressor. Although mild, these

experiences are powerful enough to cause long-term epigenetic and behavioral changes (Doherty et al., 2017; Roth, et al., 2009; Doherty, Forster, & Roth, 2015). It is logical to assume that our maltreatment condition would activate the HPA axis, thereby producing a significant difference in glucocorticoid concentrations in maltreatment-group subjects. Our study however found no significant main effects of either treatment group or of sex, nor any significant interactions between the two. These data are consistent with other work showing the lack of a corticosterone response to stressful and aversive stimuli for the first few weeks of early pup life (Loman & Gunnar, 2010). As the behavioral paradigm has been previously shown to induce maltreatment-specific deficits in behavior and changes to the epigenome, data suggest from the current study that stress-induced elevations in corticosterone are likely not responsible for these changes.

4.3 Limitations and future directions

This project had various limitations. While seeing no significant difference in corticosterone levels, this suggests our pups are protected from HPA axis activation. However, it would be wise to test our model at a later age (subjecting older pups to the same caregiving conditions), such as a few weeks after birth, when the stress hyporesponsive period no longer exists (Loman & Gunnar, 2010). This would allow us to determine that our paradigm is truly stress-inducing and that this stress hyporesponsive period exists for only a limited amount of time in early life.

The project was also limited by the lack of sample collection at other points in time. For example, if we measured corticosterone levels immediately following our caregiving paradigm, we would be able to see if there was any instant activation of the HPA axis. This could allow us to better understand the stress-hyporesponsive period in

regard to our paradigm, as well as obtain a better picture of how our paradigm interacts with the physiological stress response.

Future direction in this line of work could perhaps focus on better understanding our paradigm's effect on the brain's glucocorticoid receptors. Although in rodents two receptors exist for corticosterone, the glucocorticoid receptor has a more widespread distribution in the brain (Reul & de Kloet, 1985). As mentioned previously, multiple studies have found significant epigenomic and transcriptional changes in genes necessary for glucocorticoid receptor formation in subjects exposed to a stressful early-life event (Weaver et al., 2004; McGowan et al., 2009; Perroud et al., 2011). By using these studies as a comparison, we can create our own investigations in the effects of our paradigm. Initially, this project can begin by measuring gene expression of the glucocorticoid receptor, in various brain regions (Agba et al., 2017). Further, tissue-specific investigation of epigenetic modifications on the gene's promoter and/or exons could provide insight as to how transcription is regulated. Lastly, as the receptor is a cytosolic protein, a western blot could be utilized to approximate receptor concentration. Together, all of these methods could provide a better insight as to how our paradigm influences our subjects by investigating different brain regions as well as different developmental periods.

4.4   Conclusion

The findings in this study bring us closer to understanding how early-life stress induces behavioral and neurological outcomes later in life. For our purposes, the lack of significance of corticosterone concentrations across treatment groups suggests our behavioral paradigm has long-lasting effects into adulthood not produced by stress-induced increases in corticosterone. By studying a model of early-life adversity, we

hope to be able to provide insight into the biological consequences of development

stress, which can provide valuable information to potentially aid those in need.

REFERENCES

Abrahamson, A. C., Baker, L. A., & Caspi, A. (2002). Rebellious teens? genetic and environmental influences on the social attitudes of adolescents. *Journal of Personality and Social Psychology, 83*(6), 1392-1408.

Agba, O. B., Lausser, L., Huse, K., Bergmeier, C., Jahn, N., Groth, M., . . . Platzer, M. (2017). Tissue-, sex-, and age-specific DNA methylation of rat glucocorticoid receptor gene promoter and insulin-like growth factor 2 imprinting control region. *Physiological Genomics, 49(*11), 690-702.

Anda, R. F., Brown, D. W., Felitti, V. J., Bremner, J. D., Dube, S. R., & Giles, W. H. (2007). Adverse childhood experiences and prescribed psychotropic medications in adults. *American Journal of Preventive Medicine, 32*(5), 389-394.

Belsky, J., Jaffee, S. R., Caspi, A., Moffitt, T., & Silva, P. A. (2003). Intergenerational relationships in young adulthood and their life course, mental health, and personality correlates. *Journal of Family Psychology, 17*(4), 460-471.

Blaze, J., & Roth, T. L. (2017). Caregiver maltreatment causes altered neuronal DNA methylation in female rodents. *Development and Psychopathology, 29*(2), 477-489.

Blaze, J., Scheuing, L., & Roth, T. L. (2013). Differential methylation of genes in the medial prefrontal cortex of developing and adult rats following exposure to maltreatment or nurturing care during infancy. *Developmental Neuroscience, 35*(4), 306-316.

Brown, D. W., Anda, R. F., Felitti, V. J., Edwards, V. J., Malarcher, A. M., Croft, J. B., & Giles, W. H. (2010). Adverse childhood experiences are associated with the risk of lung cancer: A prospective cohort study. *BMC Public Health, 10*, 20.

Casavant, S. G., Cong, X., Moore, J., & Starkweather, A. (2019). Associations between preterm infant stress, epigenetic alteration, telomere length and neurodevelopmental outcomes: A systematic review. *Early Human Development, 131*, 63-74.

Chambers, J. (2017). The neurobiology of attachment: From infancy to clinical outcomes. *Psychodynamic Psychiatry, 45*(4), 542-563.

Champagne, F. A., Francis, D. D., Mar, A., & Meaney, M. J. (2003). Variations in maternal care in the rat as a mediating influence for the effects of environment on development. *Physiology & Behavior, 79*(3), 359-371.

Charbonneau, D. H., & Healy, A. M. (2005). Genetics home reference. *Journal of Consumer Health on the Internet, 9*(4), 61-68.

Davis, E. G., Humphreys, K. L., McEwen, L. M., Sacchet, M. D., Camacho, M. C., MacIsaac, J. L., . . . Gotlib, I. H. (2017). Accelerated DNA methylation age in adolescent girls: Associations with elevated diurnal cortisol and reduced hippocampal volume. *Translational Psychiatry, 7*(8), e1223.

De Kloet, E. R., Vreugdenhil, E., Oitzl, M. S., & Joels, M. (1998). Brain corticosteroid receptor balance in health and disease. *Endocrine Reviews, 19*(3), 269-301.

Doherty, T. S., Blaze, J., Keller, S. M., & Roth, T. L. (2017). Phenotypic outcomes in adolescence and adulthood in the scarcity-adversity model of low nesting resources outside the home cage. *Developmental Psychobiology, 59(*6), 703-714.

Doherty, T. S., Forster, A., & Roth, T. L. (2016). Global and gene-specific DNA methylation alterations in the adolescent amygdala and hippocampus in an animal model of caregiver maltreatment. *Behavioural Brain Research, 298*(Pt A), 55-61.

Ehrlich, M. (2002). DNA methylation in cancer: Too much, but also too little. *Oncogene, 21*(35), 5400-5413.

Ewald, E. R., Wand, G. S., Seifuddin, F., Yang, X., Tamashiro, K. L., Potash, J. B., . . . Lee, R. S. (2014). Alterations in DNA methylation of Fkbp5 as a determinant of blood-brain correlation of glucocorticoid exposure. *Psychoneuroendocrinology, 44*, 112-122.

Felitti, V. J., Anda, R. F., Nordenberg, D., Williamson, D. F., Spitz, A. M., Edwards, V., . . . Marks, J. S. (1998). Relationship of childhood abuse and household dysfunction to many of the leading causes of death in adults. the adverse childhood experiences (ACE) study. *American Journal of Preventive Medicine, 14*(4), 245-258.

Gray, J. D., Kogan, J. F., Marrocco, J., & McEwen, B. S. (2017). Genomic and epigenomic mechanisms of glucocorticoids in the brain. *Nature Reviews. Endocrinology, 13(*11), 661-673.

Greenberg, M. T., Speltz, M. L., & Deklyen, M. (1993). The role of attachment in the early development of disruptive behavior problems. *Development and Psychopathology, 5(*1-2), 191-213.

Halberg, F. (1969). Chronobiology. *Annual Review of Physiology, 31*, 675-725.

Hamilton, O. K. L., Zhang, Q., McRae, A. F., Walker, R. M., Morris, S. W., Redmond, P., . . . Marioni, R. E. (2019). An epigenetic score for BMI based on DNA methylation correlates with poor physical health and major disease in the lothian birth cohort. *International Journal of Obesity* (2005).

Heim, C., Newport, D. J., Heit, S., Graham, Y. P., Wilcox, M., Bonsall, R., . . . Nemeroff, C. B. (2000). Pituitary-adrenal and autonomic responses to stress in women after sexual and physical abuse in childhood. *Jama, 284*(5), 592-597.

Heim, C., Young, L. J., Newport, D. J., Mletzko, T., Miller, A. H., & Nemeroff, C. B. (2009). Lower CSF oxytocin concentrations in women with a history of childhood abuse. *Molecular Psychiatry, 14*(10), 954-958.

Hertsgaard, L., Gunnar, M., Erickson, M. F., & Nachmias, M. (1995). Adrenocortical responses to the strange situation in infants with disorganized/disoriented attachment relationships. *Child Development, 66*(4), 1100-1106.

Holson, R. R., & Pearce, B. (1992). Principles and pitfalls in the analysis of prenatal treatment effects in multiparous species. *Neurotoxicology and Teratology, 14*(3), 221-228.

Hunter, R. G., Gagnidze, K., McEwen, B. S., & Pfaff, D. W. (2015). Stress and the dynamic genome: Steroids, epigenetics, and the transposome. *Proceedings of the National Academy of Sciences of the United States of America, 112*(22), 6828-6833.

Kanherkar, R. R., Bhatia-Dey, N., & Csoka, A. B. (2014). Epigenetics across the human lifespan. *Frontiers in Cell and Developmental Biology, 2*, 49.

Keller, S. M., Doherty, T. S., & Roth, T. L. (2018). Pharmacological manipulation of DNA methylation in adult female rats normalizes behavioral consequences of early-life maltreatment. *Frontiers in Behavioral Neuroscience, 12*, 126.

Koss, K. J., & Gunnar, M. R. (2018). Annual research review: Early adversity, the hypothalamic-pituitary-adrenocortical axis, and child psychopathology. *Journal of Child Psychology and Psychiatry, and Allied Disciplines, 59*(4), 327-346.

Lahdepuro, A., Savolainen, K., Lahti-Pulkkinen, M., Eriksson, J. G., Lahti, J., Tuovinen, S., . . . Raikkonen, K. (2019). The impact of early life stress on anxiety symptoms in late adulthood. *Scientific Reports, 9*(1), 0.

Levine, S. (1994). The ontogeny of the hypothalamic-pituitary-adrenal axis. the influence of maternal factors. *Annals of the New York Academy of Sciences, 746*, 93.

Li, J., Chen, J., Ma, N., Yan, D., Wang, Y., Zhao, X., . . . Zhang, C. (2019). Effects of corticosterone on the expression of mature brain-derived neurotrophic factor (mBDNF) and proBDNF in the hippocampal dentate gyrus. *Behavioural Brain Research, 365*, 150-156.

Loman, M. M., Gunnar, M. R., & Early Experience, S. (2010). Early experience and the development of stress reactivity and regulation in children. *Neuroscience and Biobehavioral Reviews, 34*(6), 867-876.

Lupien, S. J., McEwen, B. S., Gunnar, M. R., & Heim, C. (2009). Effects of stress throughout the lifespan on the brain, behaviour and cognition. *Nature Reviews. Neuroscience, 10*(6), 434-445.

Manzano Nieves, G., Schilit Nitenson, A., Lee, H. I., Gallo, M., Aguilar, Z., Johnsen, A., . . . Bath, K. G. (2019). Early life stress delays sexual maturation in female mice. *Frontiers in Molecular Neuroscience, 12,* 27.

Matilainen, O., Quiros, P. M., & Auwerx, J. (2017). Mitochondria and epigenetics - crosstalk in homeostasis and stress. *Trends in Cell Biology, 27*(6), 453-463.

McGowan, P. O., Sasaki, A., D'Alessio, A. C., Dymov, S., Labonte, B., Szyf, M., . . . Meaney, M. J. (2009a). Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. *Nature Neuroscience, 12*(3), 342-348.

Meyer, J. S. (1983). Early adrenalectomy stimulates subsequent growth and development of the rat brain. *Experimental Neurology, 82*(2), 432-446.

Moore, D. S. (2015). The developing genome: An introduction to behavioral epigenetics. Oxford; New York, NY: *Oxford University Press*.

Mortada, I., & Mortada, R. (2018). Epigenetic changes in mesenchymal stem cells differentiation. *European Journal of Medical Genetics, 61*(2), 114-118.

Murgatroyd, C., Quinn, J. P., Sharp, H. M., Pickles, A., & Hill, J. (2015). Effects of prenatal and postnatal depression, and maternal stroking, at the glucocorticoid receptor gene. *Translational Psychiatry, 5*, e560.

Nettis, M. A., Pariante, C. M., & Mondelli, V. (2019). Early-life adversity, systemic inflammation and comorbid physical and psychiatric illnesses of adult life. *Current Topics in Behavioral Neurosciences*.

Pariante, C. M., & Lightman, S. L. (2008). The HPA axis in major depression: Classical theories and new developments. *Trends in Neurosciences, 31*(9), 464-468.

Perroud, N., Paoloni-Giacobino, A., Prada, P., Olie, E., Salzmann, A., Nicastro, R., . . . Malafosse, A. (2011). Increased methylation of glucocorticoid receptor gene (NR3C1) in adults with a history of childhood maltreatment: A link with the severity and type of trauma. *Translational Psychiatry, 1*, e59.

Portfors, C. V. (2007). Types and functions of ultrasonic vocalizations in laboratory rats and mice. *Journal of the American Association for Laboratory Animal Science: JAALAS, 46*(1), 28-34.

Reichardt, H. M., Tuckermann, J. P., Bauer, A., & Schutz, G. (2000). Molecular genetic dissection of glucocorticoid receptor function in vivo. *Zeitschrift Fur Rheumatologie, 59* Suppl 2, 5.

Reul, J. M., & de Kloet, E. R. (1985). Two receptor systems for corticosterone in rat brain: Microdistribution and differential occupation. *Endocrinology, 117*(6), 2505-2511.

Ridder, S., Chourbaji, S., Hellweg, R., Urani, A., Zacher, C., Schmid, W., . . . Gass, P. (2005a). Mice with genetically altered glucocorticoid receptor expression show altered sensitivity for stress-induced depressive reactions. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience, 25*(26), 6243-6250.

Romero Mdel, M., Holmgren-Holm, F., Grasa Mdel, M., Esteve, M., Remesar, X., Fernandez-Lopez, J. A., & Alemany, M. (2013). Modulation in wistar rats of blood corticosterone compartmentation by sex and a cafeteria diet. *PloS One, 8*(2), e57342.

Roth, T. L., Lubin, F. D., Funk, A. J., & Sweatt, J. D. (2009). Lasting epigenetic influence of early-life adversity on the BDNF gene. *Biological Psychiatry, 65(*9), 760-769.

Russ, T. C., Stamatakis, E., Hamer, M., Starr, J. M., Kivimaki, M., & Batty, G. D. (2012). Association between psychological distress and mortality: Individual participant pooled analysis of 10 prospective cohort studies. *BMJ (Clinical Research Ed.), 345*, e4933.

Sapolsky, R. M., & Meaney, M. J. (1986). Maturation of the adrenocortical stress response: Neuroendocrine control mechanisms and the stress hyporesponsive period. *Brain Research, 396*(1), 64-76.

Sapolsky, R. M., Romero, L. M., & Munck, A. U. (2000). How do glucocorticoids influence stress responses? integrating permissive, suppressive, stimulatory, and preparative actions. *Endocrine Reviews, 21*(1), 55-89.

Smith, M. A., Makino, S., Kvetnansky, R., & Post, R. M. (1995). Stress and glucocorticoids affect the expression of brain-derived neurotrophic factor and neurotrophin-3 mRNAs in the hippocampus. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 15*(3 Pt 1), 1768-1777.

Suomi, S. J., Harlow, H. F., & McKinney, W. T. (1972). Monkey psychiatrists. *The American Journal of Psychiatry, 128(*8), 927-932.

Ulrich-Lai, Y. M., & Herman, J. P. (2009). Neural regulation of endocrine and autonomic stress responses. *Nature Reviews. Neuroscience, 10*(6), 397-409.

Vaiserman, A. M., & Koliada, A. K. (2017). Early-life adversity and long-term neurobehavioral outcomes: Epigenome as a bridge? *Human Genomics, 11*(1), z.

Vreugdenhil, E., Verissimo, C. S., Mariman, R., Kamphorst, J. T., Barbosa, J. S., Zweers, T., . . . Fitzsimons, C. P. (2009). MicroRNA 18 and 124a down-regulate the glucocorticoid receptor: Implications for glucocorticoid responsiveness in the brain. *Endocrinology, 150*(5), 2220-2228.

Weaver, I. C., Cervoni, N., Champagne, F. A., D'Alessio, A. C., Sharma, S., Seckl, J. R., . . . Meaney, M. J. (2004). Epigenetic programming by maternal behavior. *Nature Neuroscience, 7*(8), 847-854.

Wiener, S. G., Bayart, F., Faull, K. F., & Levine, S. (1990). Behavioral and physiological responses to maternal separation in squirrel monkeys (saimiri sciureus). *Behavioral Neuroscience, 104*(1), 108-115.

Wildeman, C., Emanuel, N., Leventhal, J. M., Putnam-Hornstein, E., Waldfogel, J., & Lee, H. (2014). The prevalence of confirmed maltreatment among US children, 2004 to 2011. *JAMA Pediatrics, 168*(8), 706-713.