ASSESSMENT OF CAREGIVER EXPERIENCES AND THEIR INFLUENCE ON GLOBAL DNA METHYLATION WITHIN THE ADOLESCENT HIPPOCAMPUS AND AMYGDALA

by

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

Spring 2015

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ACKNOWLEDGMENTS

First and foremost, I would like to thank Dr. Tania Roth for being one of the most influential and exceptional mentors I have ever had. Her constant support and guidance has helped me mature both personally and as a researcher. I have learned an unbelievable amount in the past three years as an undergraduate in her lab, and I feel so fortunate and proud to be a part of this research. I will always be incredibly grateful to Dr. Roth for enabling me to have such significant experiences and for preparing me to have a successful future in research.

Furthermore, I want to thank Tiffany Doherty for being a wonderful graduate student to work with on this project. I appreciate her patience and her willingness to teach me while she herself was still troubleshooting much of the new lab work. I hope we worked out many kinks together that will make future projects run more smoothly! I learned a great deal that goes beyond the Roth Lab from Tiffany, and I am glad to say that I can take away so much from our work together.

I would also like to thank Jen Blaze for her early mentoring in the lab, training me for various protocols, and helping me acclimate to the new environment. Additionally, I want to thank all of the other Roth Lab members—both former and current—for all of their help. Of course, I must thank my amazing parents for their unwavering love and support, as well as the rest of my family and friends for theirs.

Finally, I want to acknowledge The Undergraduate Research Program, The University of Delaware Research Foundation, The University of Delaware Science and Engineering Summer Scholars Program, and grant (1P20GM103653) for their funding and support for my research.

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ABSTRACT

Current neuroscience and developmental psychology research reveals that there are sensitive postnatal periods during which the developing brain has a high level of plasticity. Therefore, early-life experiences can shape neural circuits, determining the structural and functional aspects of brain and behavior throughout the lifespan. More precisely, early-caregiver experiences can produce epigenetic modifications, which are functional and heritable changes to the genome that do not alter the DNA sequence. This study focuses specifically on DNA methylation—an epigenetic alteration that is typically associated with gene silencing and transcriptional suppression—in the ventral hippocampus, dorsal hippocampus, and amygdala. Previous research with adult animals indicates that early-life stress or experiences with a caregiver can epigenetically alter genes in these two regions. However, studies have not examined whether such effects are present during adolescence. This study aimed to quantify levels of 5-methylcytosine (5-mC) within the genome of adolescent rats that were exposed to various caregiving experiences (aversive vs. nurturing) during the first postnatal week of life. Results indicate that exposure to aversive caregiving was associated with significantly higher 5-mC levels in the dorsal hippocampus, and this effect was only present in males. Maltreated males in comparison to maltreated females had higher methylation in the ventral hippocampus, but they did not differ from nurtured controls. Group differences in 5-mC levels were not observed in the amygdala. Together, these data empirically support the hypothesis that early-life caregiver experiences differentially affect the epigenome, and that these effects are present in specific regions of the adolescent brain.

Chapter 1

INTRODUCTION

1.1 Childhood Maltreatment

Early-life adversity has long-lasting and even transgenerational effects on neurodevelopment and behavior. Childhood maltreatment is a serious and prevalent problem in today's society that threatens the safety and welfare of children worldwide. By definition, maltreatment includes physical abuse, sexual abuse, psychological or emotional abuse, and neglect (Cicchetti & Toth, 2005). In 1989, the United Nations General Assembly adopted the Convention on the Rights of the Child (CRC), which is a human rights treaty outlining the multifaceted rights of children. The CRC went into effect one year later, after being ratified by the required number of nations. To date, 194 countries and every United Nations member state have ratified the CRC, with the exception of Somalia and the United States of America (Cicchetti & Toth, 1993; Convention on the Rights of the Child, 1989). The failure of the United States—one of the most powerful and influential nations in the world—to support a treaty outlining the fundamental rights and dignities of its children is alarming. In fact, it provokes the debate of whether or not the welfare of American children is truly a priority.

In 2013, U.S. government statistics reported an estimated 3.5 million referrals concerning the wellbeing of 6.4 million children to Child Protective Services (CPS). Over two million reports were accepted and investigated, and CPS determined approximately 679,000 child abuse and neglect victims from these reports (U.S. Department of Health and Human Services, 2015). These are startling numbers and

these reports only represent the investigated cases referred to CPS; therefore, the actual number of maltreated children is likely to be much higher (Cicchetti & Toth, 2005).

The reported statistics are quite concerning, given that traumatic experiences with a parent or caregiver early in life can have profound effects on cognitive and emotional development (Cicchetti, 2003; Fernald & Gunnar, 2009). After all, infancy and childhood are periods of postnatal life that are sensitive to environmental experiences. Previous human research suggests that nurturing early-life maternal care is associated with resilience to psychological disorders, while adverse conditions experienced early in life—such as maternal depression, maternal substance abuse, and poverty—have been associated with vulnerability to psychopathology later in life (Korosi & Baram, 2009). To further elaborate, there is a substantial amount of evidence associating reported childhood maltreatment with an increased risk of developing anxiety, depression, and other psychopathologies such as post-traumatic stress disorder (PTSD) (Osofsky & Scheeringa, 1997). For example, a meta-analysis by Nanni, Uher, and Danese (2012) indicated that individuals who had a history of childhood maltreatment were twice as likely to develop recurrent and persistent depressive episodes than individuals who did not. When taken into consideration together, current research provides an overwhelming amount of support to the association between childhood maltreatment and long-term mental health outcomes.

1.2 Adolescence as a Sensitive Developmental Period

Although human development is a continuous process, certain time points are considered more influential than others. Adolescence is one of these crucial developmental periods and Holmbeck and Updegrove (1995) noted that adolescence is

characterized by more biological, psychological, and social role changes than any other life stages, with the exception of infancy. Generally, there are three major types of changes that occur during adolescence that challenge an individual—physiological, cognitive, and psychological or emotional changes—and failure to effectively cope with these challenges may result in susceptibility to psychopathology later in life (Archer, 2005).

Puberty is a universal developmental experience that can be thought of as the process of becoming physically and sexually mature in the transition from childhood to adulthood. This transformation is initiated by changes in endocrine activity such as an increase in hormone production by glands involved in sexual maturation (Archer, 2005). These hormones not only cause physical changes like growth spurts, underarm and pubic hair growth, and maturation of genitalia, but they also influence neurological development, which in turn affects cognition, psychology, and emotional responses (Nelson, Leibenluft, McClure, & Pine, 2005; Spear, 2003). Adolescence is a marked period where humans develop a higher level of thinking and are able to comprehend abstract ideas and concepts (Archer, 2005). Since cognitive function and reasonableness are closely associated with emotional responsivity and psychological thinking patterns (Cowan, 1982), it makes sense that these processes would be affected by the cognitive and hormonal changes occurring during adolescence. This period of development is typically known for an increase in emotional reactivity (Guyer et al., 2008; Pfeifer et al., 2011), moodiness (Buchanan, Eccles, & Becker, 1992), and risky behavior (Steinberg, 2008).

Although there is a general chronological sequence for adolescent development, one of the most noticeable differences lies in the rate of maturation and

the onset of puberty between the sexes as well as individually within genders. Despite the variability of pubertal developmental stages seen in individuals at the same chronological age, on average, females begin to mature earlier than males do (Archer, 2005). For this reason, it is especially critical to include subjects of both sexes when studying adolescence.

Building upon the perinatal sexual differentiation (Collaer & Hines, 1995), the gonadal steroid hormone action during adolescence is fundamental for shaping brain development and reorganization. The long-lasting modifications in neural circuitry are both sex- and region-specific, like volumetric changes seen in the amygdala and hippocampus during adolescence (Sisk & Zehr, 2005). In humans, males showed a more pronounced increase in amygdala volume during this time point whereas enlargement of the hippocampus was more prominent in females (Giedd, Castellanos, Rajapakse, Vaituzis, & Rapoport, 1997). These structures play a large role in determining adult behavioral responses and maturity.

With so many changes occurring simultaneously, it is easy to understand why adolescents seem to be particularly vulnerable to stress, which could have potential long-term consequences due to the developmental neural restructuring taking place (Spear, 2003). Since adolescence is an especially sensitive time for hormone-dependent organization, it is also a period of particular vulnerability to psychopathologies, such as eating disorders and depression (Sisk & Zehr, 2005). Even studies using rodent models have identified adolescence as a vulnerable period to alter behavior and neurodevelopment (Schneider, 2013). Just as in humans, the onset of adolescence in rats is a difficult period to pinpoint exactly; however, some developmental milestones can be used to estimate when this period is occurring.

Weaning the pups from the biological mother generally occurs during the fourth week of life, puberty onset occurs around postnatal day 35-38 in female rats and 38-45 in males, and young adulthood is typically considered to begin when rats have reached sexual maturity, at approximately 60 days of age (Green & McCormick, 2013; Schneider, 2013).

Many studies have exposed adolescent rats to stressors and subsequently observed changes in learning and memory as well as depressive and anxious behaviors during adulthood (Green & McCormick, 2013; McCormick & Green, 2013). However, fewer studies focus on the changes already occurring during adolescence following early-life stress, and these changes could be implicated in the increased susceptibility for psychiatric illness development often observed during this time of life (Paus, Keshavan, & Giedd, 2008).

1.3 Role of the Limbic System in Stress Response

The limbic system is a bidirectional neural network that functions as the brain's emotional processing system. As one of the most basic evolutionary survival networks, the structures and pathways involved in the limbic system process emotional reactions and responses to both aversive and positive stimuli (Noback, Strominger, Demarest, & Ruggiero, 2005). Although many cortical areas are connected to this circuitry, the major limbic structures are the amygdala, the hippocampus, and the mesocortex, of which the prefrontal cortex plays a major role. The amygdala emerges as the defining hub and nodal center of the limbic system; it couples highly processed sensory information from the neocortical and mesocortical areas with the subcortical nuclei of the circuitry and transmits consolidated information to the thalamus, hypothalamus, and brainstem, where emotional responses

are evoked (Noback et al., 2005). One important connection that the amygdala makes is with the hippocampus—a structure that plays a principal role in learning and memory. It has been previously established that the hippocampus is functionally localized and specific; the dorsal region is implicated in spatial-related behaviors and the ventral region is associated with motivation- and emotion-related behaviors (Kim & Jung, 2006).

The different roles of the amygdala and hippocampus allow them to operate independently of one another, but they also communicate in significant and subtle ways. Previous research suggests that the amygdala is able to enhance the encoding, consolidation, and retention of hippocampal-dependent episodic memory so that emotional events receive priority. Studies using animal models have suggested that this modulation occurs through the actions of stress hormones activated by limbic regulation of the hypothalamic-pituitary-adrenocortical (HPA) axis (Jankord & Herman, 2008; Phelps, 2004).

The HPA axis is a neuroendocrine stress response system evolutionarily developed to maximize survival potential when faced with a challenge. Briefly, the paraventricular nucleus of the hypothalamus releases corticotropin-releasing hormone (CRH) when activated, stimulating the secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland into the bloodstream. Once the ACTH reaches the adrenal gland, the adrenal cortex produces glucocorticoids (such as cortisol), which then act on multiple organ systems, including the brain (Lightman & Conway-Campbell, 2010). When exposed to acute stress, the HPA axis activation is evolutionarily adaptive, as phasic activation helps the animal respond physiologically and behaviorally to immediate threats and challenges. However, when the HPA axis is

chronically activated due to repeated exposure to stressors, the physiological response to stress is hyperreactive, excessively excreting cortisol, and this can increase the risk of stress-related disease such as depression and anxiety (Jankord & Herman, 2008).

Human research has shown that prolonged HPA hyperreactivity due to chronic stressors early in life may lead to blunted stress response later on (Carpenter et al., 2007; Carpenter, Shattuck, Tyrka, Geracioti, & Price, 2011; Carpenter et al., 2009; Tyrka et al., 2008), and this is evident in animal models also (Huot, Gonzalez, Ladd, Thrivikraman, & Plotsky, 2004; Plotsky & Meaney, 1993; Plotsky et al., 2005). Because the brain is still developing with relatively high levels of plasticity during infancy and childhood, stress experienced during this time in life can alter HPA axis development and cause long-lasting changes in stress responsivity during adulthood (Murgatroyd & Spengler, 2011). The neural circuitry connecting the HPA axis with the limbic system allows for high levels of stress to induce changes in the hippocampus and amygdala as well. Both of these structures are especially vulnerable to high levels of stress due to their respective roles in the limbic system and also the presence of a dense number of glucocorticoid receptors (Herman, Patel, Akil, & Watson, 1989; Wang et al. 2014). Clinical studies suggest early-life stress to be associated with decreased hippocampus and amygdala volume (Driessen et al., 2000; Hanson et al., 2015; Vythilingam et al., 2002) when compared to normal control patients. Additionally, animal studies have implicated the effect of early life stressors in the inhibition of hippocampal neurogenesis (Korosi et al., 2012; Lajud, Roque, Cajero, Gutiérrez-Ospina, & Torner, 2012; Mirescu, Peters, & Gould, 2004), in impaired long-term potentiation (Ivy et al., 2010), and in alteration of amygdalar circuitry, which can increase the risk for later psychopathology (Cohen et al., 2013).

1.4 Rodent Models of Early-life Adversity

While the results of human studies are more directly translatable to the field of child maltreatment, this research contains several confounding variables that potentially influence results and conclusions. Therefore, it is important to develop animal models in which early environmental conditions can be manipulated in a controlled and regulated manner to optimize studying gene-environment interactions. Using animal subjects allows researchers to control for genetic background and also allows for the utilization of intrusive techniques to assess biological changes in specific brain regions. Additionally, rodent studies have been able to replicate and extend many of the findings from human and non-human primate research, which validates the use of these models. Several paradigms of early-life adversity are reported in the literature, of which many manipulate the mother-pup relationship to investigate long-term consequences on behavior as well as brain structure and function of the offspring.

Of particular relevance to the current study are the experimental designs utilizing resource deprivation to provoke unpredictable and aversive maternal behavior from rodent mothers towards offspring (Ivy, Brunson, Sandman, & Baram, 2008; Roth, Lubin, Funk, & Sweatt, 2009; Roth & Sullivan, 2005). By depriving the dams of nesting material, the mothers display higher rates of fragmented nurturing care (such as nursing, licking, and grooming) towards the pups (Ivy et al., 2008). The amount of nurturing caregiving behaviors an infant rat experiences has been linked by Michael Meaney and his colleagues (Meaney & Szyf, 2005; Szyf, Weaver, Champagne, Diorio, & Meaney, 2005; Szyf, Weaver, & Meaney, 2007; Weaver, Diorio, Seckl, Szyf, & Meaney, 2004; Zhang, Parent, Weaver, & Meaney, 2004) to epigenetic changes that catalyze differences in HPA axis responsiveness. For example, rat

mothers exhibiting high levels of nurturing behaviors had offspring with less DNA methylation of the *glucocorticoid receptor (GR)* gene promoter in the hippocampus when compared to the offspring of mothers that exhibited low levels of nurturing care (Weaver et al., 2004). Rodent models have enabled researchers to associate early-life caregiver experiences with specific epigenetic changes in various brain regions—which is much more difficult to do with human studies.

1.5 DNA Methylation as a Regulatory Epigenetic Mechanism

The term 'epigenetics', coined by biologist and geneticist Conrad Waddington in 1942, literally means "above the genome." In today's research, epigenetic modifications are being investigated as potential mechanisms for how environmental experiences interact with genes without altering the DNA sequence. Epigenetic alterations are proposed to cause changes in neural function and behavior by responding dynamically to events that occur throughout development, enabling both immediate and possibly persistent changes in gene activity. One mechanism by which this can occur is through DNA methylation, where methyl groups (CH_3) are added to the 5'-position of cytosine rings in cytosine-guanine (CpG) dinucleotides. Areas of the genome rich with CpG sites are termed 'CpG islands', and the DNA methylation of these areas is typically associated with transcriptional repression (Bird, 2002). Enzymes called DNA methyltransferases (DNMTs) catalyze the addition of the methyl group to the CpG dinucleotide. When CpG islands are heavily methylated, the DNA develops higher-affinity interactions with the histone around which it is wrapped, which then condenses the complex. Methylation also typically disrupts transcription factor binding and therefore gene expression (Jaenisch & Bird, 2003; Klose & Bird, 2006).

When studying gene-environment interactions, DNA methylation has been the most studied of epigenetic mechanisms, due to its stable and even hereditary nature regarding gene regulation. Current research is discovering patterns of DNA methylation within brain regions (Simmons, Stringfellow, Glover, Wagle, & Clinton, 2013), across various genes (Ehrlich, 2003), throughout development (Takasugi, 2011), and in connection to psychiatric disorders (McGowan & Szyf, 2010). While DNA methylation can regulate heritable, long-term gene expression, it can also cause transient changes in gene activity in response to postnatal environmental experiences (Roth, 2012).

Although much of the existing evidence focuses on methylation patterns of specific genes, emerging research is beginning to characterize genome-wide levels of DNA methylation. Clinical studies are linking such global methylation differences to pathologies, such as coronary artery disease (Sharma et al., 2008) and various cancers, emphasizing the potential oncological advantages of genome-wide methylation profiling in tumor aggressiveness and disease progression (Keita et al., 2013). Changes in global levels of 5-methylcytosine DNA are also evident in post-mortem brain tissue of Alzheimer's disease patients (Chouliaras et al., 2013; Coppieters et al., 2014) and in peripheral blood samples of suicidal psychiatric patients (Murphy et al., 2013) when compared to controls, associating aberrant levels of DNA methylation with neurological disorders and mental illness.

1.6 Rationale for Current Research

The majority of existing research relevant to the current investigation has focused on gene-specific epigenetic modifications following early-life stress (McGowan et al., 2009; Murgatroyd et al., 2009; Roth et al., 2009). This includes a

recent study by Roth, Matt, Chen, & Blaze (2014), which examined differences in DNA methylation of the *Bdnf* gene in the infant and adult hippocampus and amygdala following the same early-life caregiver experience model used in the current study. However, accumulating evidence indicates that DNA methylation outside of CpG islands is perhaps equally as important in regulating genomic function (Irizarry et al., 2009; Shen, Chow, Wang, & Fan, 2006). This is why it is also important to investigate changes observed in global levels of DNA methylation following early-life stress. Moreover, many potential pharmacological treatments targeting cytosine methylation are nonspecific, acting at a genome-wide level (Syzf, 2003). These nonspecific actions could have broad repercussions affecting other diseases and psychiatric disorders, since most have their own unique DNA methylation patterns.

Additionally, current literature typically focuses on the immediate effects of early-life adversity (during infancy) or the long-term consequences (seen during adulthood), but there is surprisingly little research exploring the effects observed during adolescence. As previously discussed, adolescence is a sensitive period of development, and epigenetic alterations produced by early-life stress could have relevance to the physiological, cognitive, and psychological changes faced in adolescence. Further, neural changes occurring during this time point may be causing the observed gender differences in emotional reactivity in response to stressors and the subsequent moderation of depressive symptoms (Charbonneau, Mezulis, & Hyde, 2009; Hare et al., 2008). All things considered, the importance of observing developmental changes in both sexes is evident as well. All too often in the literature, studies use only male subjects to eliminate the potentially confounding variable of the female estrous cycle. However, as previously discussed, stress responses vary

considerably between males and females, and it is therefore important to look at differences observed in both sexes (McCarthy et al., 2009).

In this study, a limited bedding model (Roth et al., 2009) was used to create stressful conditions for a dam caring for a foster litter instead of a maternal separation regimen, in an attempt to better replicate an abusive or neglectful caregiver experience in humans. A hallmark of maternal behavior in childhood maltreatment cases is its fragmented quality and unpredictability (Ivy et al., 2008). Furthermore, since the first postnatal week of life is decisive for appropriate development of stress response and the HPA axis of rats, this is the time period in which the infant rats were exposed to adverse or nurturing caregiving environments.

The current study investigates the hypotheses that: 1) early-life exposure to nurturing or adverse caregiving environments produces distinct genome-wide epigenetic markings during adolescence present in both the dorsal and ventral hippocampus, as well as in the amygdala; and, 2) these epigenetic patterns will likely vary between sexes.

Chapter 2

METHODS

2.1 Subjects

In this study, outbred Long-Evans rats obtained from Harlan were housed in polypropylene cages (18”x9”x8”) with plenty of wood shavings to use as bedding. Animals were housed in a temperature- and light-controlled colony room (12 hour light/dark cycle, with lights on at 6:00 am) and had access to food and water *ad libitum*. All experimental procedures were performed during the light cycle. Dams were bred in the laboratory and gave birth to at least one litter prior to their use in any experiments to ensure that no first-time mothers were used in the study. Long-Evans rats were chosen for this study because of their tendency to exhibit more nurturing maternal behaviors when compared to other rodent species (McIver & Jeffrey, 1967). Postnatal day (PN) 0 was designated as the day the pups were born, and on PN1, litters were culled to 5-6 males and 5-6 females. The University of Delaware Animal Care and Use Committee approved of all procedures prior to the execution of the experiment.

2.2 Caregiving Manipulations

Using the within-litter design previously established (Blaze, Scheuing, & Roth, 2013; Roth et al., 2009; Roth & Sullivan, 2005), infant rats of both sexes were repeatedly exposed to one of three conditions: maltreatment, cross-fostered care, or normal maternal care. These exposures occurred for thirty minutes per day throughout

the first postnatal week (PN1-PN7), during which dam and pup behaviors were observed and recorded. Before each exposure, every pup was weighed and marked for identification, and each condition ideally contained two male pups and two female pups. In the maltreatment condition, a lactating female was placed in a novel environment that contained limited nesting resources (only a handful of wood shavings for nesting) and was given insufficient time (only five minutes) to habituate to her environment before she received up to four experimental pups. The new environment consisted of a black plastic container (18”x12”x18”) and was located in a room separate from the rat colony. The inadequate habituation time to the novel environment and insufficient nesting material were stressful conditions for the rat mother, as she tended to exhibit more aversive and neglectful behaviors towards the pups (these will be described more below). The littermates (up to four) in the cross-fostered care condition were also exposed to a lactating female, but this female was given one hour to habituate to the novel environment (18”x12”x18” black plastic container) and was provided plenty of wood shavings for nesting material (an approximate 2 cm layer across the chamber floor) to facilitate nurturing behaviors. Therefore, this condition provided exposure to a positive caregiving environment—unlike the maltreatment condition—and controlled for the stress effects of being taken away from the biological mother and placed with another caregiver. The remaining littermates served as normal maternal care controls, and they were left in the home cage with their biological mother after being weighed and marked for identification. The biological mother provided a positive and nurturing caregiving environment for the pups. After the 30-minute sessions, experimental pups were removed from the test chamber and returned to the home cage with their biological mother. Likewise,

stimulus dams (maltreatment and cross-foster) were reunited with their biological litters immediately after each exposure session. All experimental chambers were kept between 24-29°C, as measured by a digital thermometer, to help maintain pup body temperature. The separate room where the cross-fostered and maltreatment experimental exposures took place was equipped with white noise generators to eliminate external noises. After the last exposure on PN7, all pups were left undisturbed in the home cage with their biological mother until PN21-23 when they were weaned and housed in same-sex pairs until adolescence (PN30). Once the pups reached adolescence, brains were removed.

2.3 Behavior and Vocalization Observations

During every caregiving manipulation session, audible vocalizations were recorded using a SONY audio recording device and ultrasonic vocalizations (at a frequency of 40 kHz) were recorded using an additional bat detector (Batbox III D, NHBS Ltd., UK). Ultrasonic pup vocalizations emitted at a frequency of 40 kHz are used as a distress measure in stressful situations, such as prolonged maternal separation (Hofer, 1996; Portfors, 2007). Caregiver-infant interaction behavior was recorded using a SONY video camera or live observation. The digital recordings were then transferred onto a computer, and trained research assistants assisted with the coding of the vocalizations and behavior. Both the vocalization recordings and the behavior videos were coded by two different research assistants; these scores were then averaged. Vocalizations were documented if a pup vocalization was heard during any one-minute interval throughout every 30-minute exposure session. The frequency of vocalizations occurring was then averaged across the experimental week for all three conditions individually. Caregiver and infant behavior was observed during five-

minute intervals, in which coders recorded all behaviors observed during the 30-minute session. Observed nurturing behaviors included pup licking, anogenital licking of the pups, hovering over the pups, and nursing. Behaviors considered to be aversive were rough handling, dragging, stepping on, actively avoiding, or dropping the pups. Any other observed maternal or pup behaviors—such as digging and wall-climbing—were considered neutral behaviors and were not used when determining the frequency of pup-directed nurturing or aversive caregiving behaviors. Videos were scored by trained research assistants and the observations were averaged across the seven exposure days.

2.4 Biochemical Assays

Adolescent rats were anesthetized at baseline conditions (i.e. removed from their home cage with minimal disturbance) on PN30 using isofluorane, and then brains were removed and sliced using a 1 mm brain matrix. The slices were subsequently flash frozen on untreated slides using 2-methylbutane and placed in a -80°C freezer until later processing. The ventral hippocampus, dorsal hippocampus, and amygdala (homogenate consisting of the basolateral, lateral, and central nuclei) were then individually dissected on dry ice using stereotaxic coordinates (Paxinos & Watson, 2007). Immediately after tissue removal, DNA was extracted using an Allprep DNA/RNA kit (Qiagen Inc., Valencia, CA). Purified DNA samples were analyzed for quantification by measuring the concentration and quality of nucleic acid with a NanoDrop Spectrophotometer (2000). Labeled aliquots containing purified extracted DNA samples were then stored in a -80°C freezer until later methylation assessment.

Extracted DNA samples from each of the three brain regions were diluted with RNase-free water to obtain a concentration of 25 ng/ml. Once each sample was diluted

to this concentration, MethylFlash™ Methylated DNA Quantification Kits were used to quantify genome-wide methylation levels according to the manufacturer's instructions (Epigentek, Brooklyn, NY). In this assay, 5-methylcytosine antibody recognizes 5-methylcytosines (5-mC) in genomic DNA, and the DNA is colorimetrically quantified against a standard curve. Positive (methylated) and negative (unmethylated) control DNA for the standard curve was supplied with the kit. Absorbance was measured using the Infinite ® F50 microplate reader (Tecan, Männedorf, Switzerland). All replicates fell under a 30% coefficient of variation and all standard curves had an R^2-value of 0.9 or greater. The amount of 5-mC DNA is proportional to the OD intensity and was calculated as follows: 5-mC DNA (ng) = [OD (sample – blank)/ 2 x slope, where OD is optical density, blank is buffer without DNA, and slope is the slope (OD/ng) of the standard curve using linear regression. Using this calculated value, the percentage of 5-mC content in total DNA was accurately determined using the following equation: 5-mC DNA % = (ng of 5-mC DNA/ng of total DNA in sample) x 100%. This assay accounts for methylation of all cytosines irrespective of whether they are located in promoter or non-promoter regions of the genome. Cytosine methylation in both promoter and intragenic regions are currently recognized for their role in regulating gene activity (Fazzari & Greally, 2004).

2.5 Statistical Analysis

Two-way ANOVAs, two-tailed unpaired t-tests, and Bonferroni-corrected t-tests were used to analyze data (comparing 5-mC levels across and between groups). Significance was set at $p \leq 0.05$ for all analyses. The computer program PRISM was used to help perform statistical tests and aid in the creation of graphs. Samples that

had a coefficient of variation greater than 30% were excluded (ventral hippocampus: 1 normal female and 1 maltreatment female; amygdala: 1 maltreatment male, 1 normal male, and 1 normal female). Outliers that fell more than two standard deviations above or below the mean were excluded (amygdala: 1 maltreatment male).

Chapter 3

RESULTS

3.1 Caregiver Behaviors

Caregiving and pup vocalization data for this cohort have been previously published by Blaze et al. in 2013 and Roth et al. in 2014. Data show that infants in the foster and normal care conditions experienced high levels of nurturing care (occurrence levels greater than 75%) and low levels of adversity (less than 25%), and these two groups did not differ significantly from one another (p's>0.05). In contrast, maltreated infants experienced high levels of adversity (greater than 50%) and low levels of nurturing care (less than 40%) (p's<0.001 maltreatment vs. cross-fostered or normal maternal care).

3.2 Infant Responses to Caregiving Conditions

Audible and ultrasonic (40 kHz) vocalizations of male and female infant rats in the three caregiver conditions were recorded during each exposure session, and these results likewise have been published previously (Blaze et al., 2013; Roth et al., 2014). Results show that maltreated infant rats emitted significantly more audible (occurrence levels greater than 50%) and ultrasonic vocalizations (greater than 75%) when compared to normal care ($p<0.05$ audible, $p<0.001$ ultrasonic), and foster care ($p<0.01$ audible, $p<0.001$ ultrasonic) rats. There were no significant differences in emitted audible or ultrasonic vocalizations between the normal and foster care groups (p's>0.05).

3.3 DNA Methylation of the Ventral Hippocampus in Adolescence

Global levels of 5-mC were first quantified in adolescent (PN30) ventral hippocampus tissue (Figure 1). A two-way ANOVA revealed that in the ventral hippocampus, there was a main effect of sex ($F_{1,52}$=4.218, $p<0.05$), but no main effect of infant condition ($F_{2,52}$=0.2207, p=0.8027). There was also a significant interaction between infant condition × sex ($F_{2,52}$=3.311, $p<0.05$). Maltreated males had significantly higher levels of methylated DNA in comparison to maltreated females (t=2.98, $p<0.05$). There were no significant differences in levels of methylated DNA in the other two infant conditions (all p's>0.05).

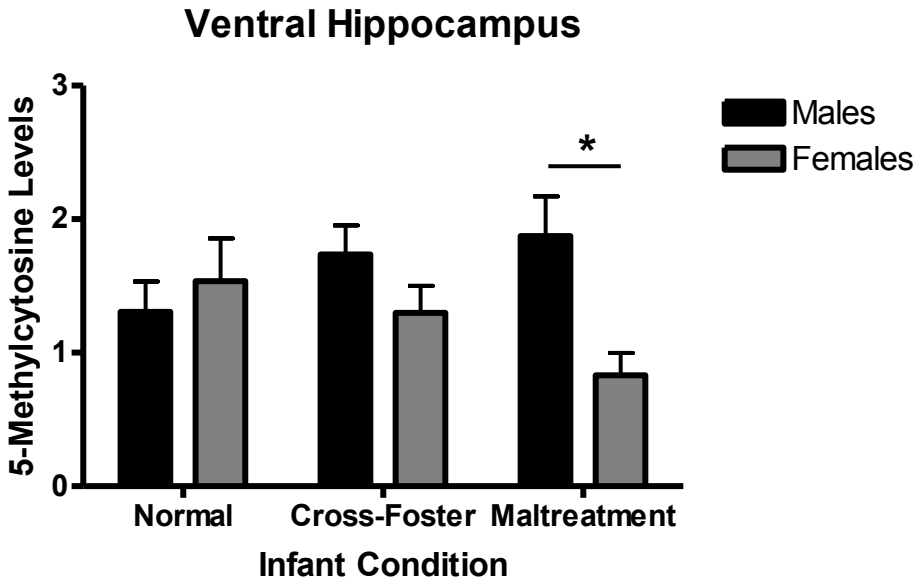

Figure 1. Global levels of 5-methlycytosine DNA in adolescent (PN30) ventral hippocampus tissue. Results indicate that maltreated males had significantly higher levels of DNA methylation than maltreated females (*$p<0.05$). n=9-10/group; subjects derived from 10 litters; error bars=SEM.

3.4 DNA Methylations of the Dorsal Hippocampus in Adolescence

Global levels of 5-mC were quantified for dorsal hippocampus tissue (Figure 2). A two-way ANOVA revealed that in the dorsal hippocampus, there was a main effect of infant condition ($F_{2,54}$=3.537, $p<0.05$), no main effect of sex ($F_{1,54}$=4.010, $p=0.0503$), and a significant interaction between infant condition × sex ($F_{2,54}$=3.599, $p<0.05$). Post-hoc testing showed that maltreated males had significantly higher levels of methylated DNA in comparison to cross-fostered ($t=2.866$, $p<0.05$) and normal care males ($t=3.015$, $p<0.01$). Furthermore, maltreated males had significantly higher levels of methylated DNA in comparison to maltreated females ($t=3.294$, $p<0.01$). There were no significant differences in levels of methylated DNA in the other two infant conditions when comparing sexes (all p's>0.05).

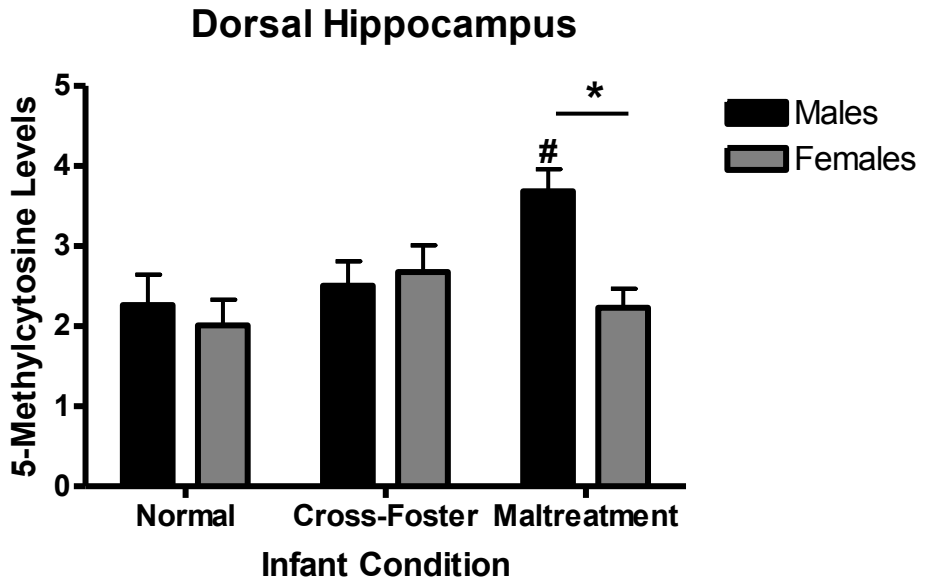

Figure 2. Global levels of 5-methlycytosine DNA in adolescent (PN30) dorsal hippocampus tissue. Results indicate that maltreated males had significantly higher levels of DNA methylation when compared to male cross-fostered and normal care controls (#$p<0.05$). Maltreated males also had significantly higher levels of 5-mC DNA when compared to maltreated females (*$p<0.05$). n=9-11/group; subjects derived from 10 litters; error bars=SEM.

3.5 DNA Methylation of the Amygdala in Adolescence

Finally, global levels of 5-mC were quantified in the adolescent amygdala tissue (Figure 3). A two-way ANOVA revealed that there was no main effect of infant condition ($F_{2,51}=2.272$, $p=0.1135$), no main effect of sex ($F_{1,51}=1.821$, $p=0.1832$), and no significant interaction between infant condition × sex ($F_{2,51}=1.525$, $p=0.2274$).

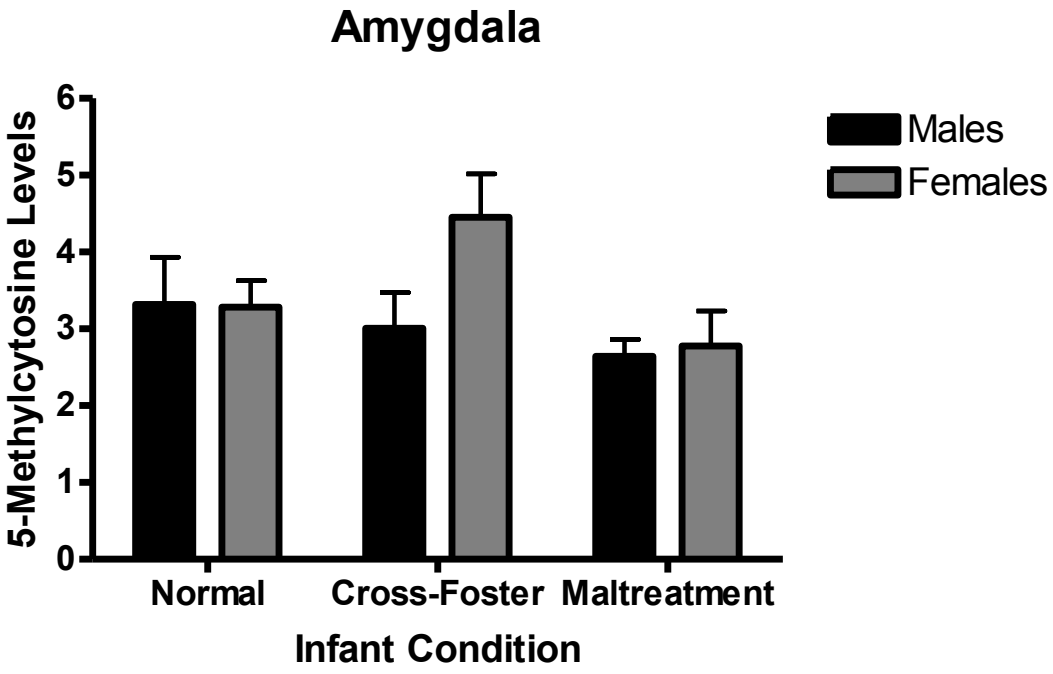

Figure 3. Global levels of 5-methlycytosine DNA in adolescent (PN30) amygdala tissue. Results indicate that there were no significant differences in DNA methylation across infant conditions and between sexes. n=8-11/group; subjects derived from 10 litters; error bars=SEM.

Chapter 4

DISCUSSION

4.1 Caregiver Behaviors and Infant Responses to Caregiving Conditions Differ Significantly Across Treatment Groups

This study aimed to investigate a link between caregiving experiences during infancy and global 5-mC levels in adolescence. This was assessed in PN30 rats of both sexes using a within-litter model that assigned different, recurring treatments to neonates throughout their first week of life. These infant rats experienced adversity outside of the homecage, which provides a unique way of studying the effect of caregiving early in life without the confounding variables of nutrient and warmth deprivation. One other strength of this study is the use of a second control group—the cross-fostered rats—in addition to the normal care controls; this allows for discrimination of effects produced by removal from the homecage/biological mother and exposure to a novel environment/caregiver from the effects produced by caregiver maltreatment.

High levels of aversive pup-directed behaviors were elicited by the dams in the maltreatment group as a result of restricting the lactating female's nesting material in a novel environment (Blaze et al., 2013; Roth et al., 2014). When comparing these behaviors to those observed in the two control groups, it was found that the control females exhibited high levels of nurturing care, validating the use of cross-fostered pups as a second control group (Blaze et al., 2013; Roth et al., 2014). It is also important to note that the infants responded to the differences in quality of care

accordingly; the maltreated pups emitted more audible and ultrasonic distress vocalizations, when compared to pups receiving more nurturing care (cross-foster and normal care) (Blaze et al., 2013; Roth et al., 2014).

These data are concordant with previous research from our lab and also complement other studies demonstrating that resource deprivation within the homecage can produce aversive caregiving (Ivy et al., 2008; Roth et al., 2014; Roth & Sullivan, 2005).

4.2 Global Levels of DNA Methylation are Influenced by Early-Life Caregiving Experiences

To quantify the effects of early-life caregiver experience on 5-mC levels, biochemical assays were performed and revealed differences that varied between brain region, sexes, and treatment groups. Focusing first on the hippocampus, some interesting differences were observed in both the ventral and the dorsal regions. In the ventral hippocampus, there was no main effect of infant condition. However, sex-specific differences were evident within the maltreatment group, where males had significantly higher levels of DNA methylation compared to the female pups in the same condition. This same sex difference in the maltreated pups is also seen in the dorsal hippocampus tissue, in addition to an increase in global methylation observed in maltreated males compared to the males in the two control groups (cross-foster and normal care). These sex-specific patterns could reflect a number of factors including differential quality of treatment received by male and female infants from the dams within each condition, as it has been previously shown that rat mothers preferentially lick and groom male pups (Moore & Morelli, 1979). Along these lines, altering the gender composition of a litter can also induce changes in caregiving behaviors and

produce epigenetic differences seen in adolescent rats (Kosten, Huang, & Nielsen, 2014). Therefore, it is important in future studies to distinguish male- vs. female-directed caregiver behaviors in this model.

The changes seen in the maltreated offspring of the current study differ from those of a previous study that showed an increase in global methylation levels in the adult dorsal hippocampus of offspring who experienced high nurturing care relative to litters who received low levels of nurturing care (Brown, Weaver, Meaney, & Szyf, 2008). However, Brown and his colleagues analyzed the tissue subregionally—separating the dorsal hippocampus further into CA1, CA2, CA3, and dentate gyrus—and at a different developmental time point than the current study (adulthood versus adolescence), which could potentially explain why the observed changes seem to contradict one another.

When global levels of methylation were quantified in the amygdala, there were no significant differences observed between infant conditions or sexes. This could be because the amygdala is comprised of various nuclei that serve distinctive functions (Pitkänen, Savander, & LeDoux, 1997), and even the right and left amygdalae have been shown to be functionally different (Markowitsch, 1998). For this assay, a homogenate composed of left and right nuclei was used, so perhaps if the amygdalar nuclei were isolated and quantified separately from one another and/or the amygdala was separated laterally, differences in DNA methylation may have been seen. It is also important to keep in mind that experience-induced changes can appear and disappear at various developmental time points (Schwarz, Nugent, & McCarthy, 2010).

4.3 Genome-Wide Methylation Patterns Could Have Implications on Behavioral Differences

This project focuses specifically on biochemical epigenetic alterations following early-life aversive care, without investigating the consequences these changes may have on neural function and/or behavior. However, many studies have correlated DNA methylation with behavioral differences following early-life stress, though most of these correlations have been made in adult animals (Anier et al., 2014; Szyf et al., 2005; Zhang et al., 2010). In general, aberrant methylation (typically increased) in the hippocampus and frontal cortex is correlated with behavioral abnormalities, such as locomotor hyperactivity and deficits in social interaction (Dong et al., 2015). The majority of the research focuses on gene-specific methylation patterns and behavior; however, some studies have examined global levels of DNA methylation. For example, Mychasiuk, Ilnytskyy, Kovalchuk, Kolb, & Gibb (2011) investigated the effects of prenatal stress intensity on the developing brain by measuring global methylation in the frontal cortex and hippocampus of 21-day-old offspring, as well as subjecting them to early behavioral testing (negative geotaxis and open field tests). They found that offspring exposed to mild prenatal stress improved at the same rate in negative geotaxis compared to controls, but highly stressed pups did not and therefore demonstrated overall deficits in comparison to controls. Sex differences were found in open field testing for the mildly stressed offspring—males displayed an increase in activity and females showed decreased activity when compared to controls— but the highly stressed pups all showed a decrease in open field activity. When measuring global DNA methylation levels, in the frontal cortex, all offspring of the highly stressed group showed a decrease in methylation, whereas in the mildly prenatally stressed group, males showed an increase and females showed

no difference when compared to controls. No sex differences were seen in the hippocampus where the mildly stressed pups demonstrated an increase in methylation and the highly stressed had decreased levels when compared to controls. This study demonstrates the stressor- and sex-specific epigenetic and phenotypic changes that can occur following gestational stress.

Although there have been numerous studies investigating the behavioral changes seen following-early life stress in accordance with observed epigenetic changes (both gene-specific and genome-wide), most of the current literature focuses on adulthood. There is a surprisingly scarce amount of research focusing on the effects of early-life stress and caregiving environment on both changes in adolescent tissue and behavior. One study by Chocyk et al. (2014) found that maternal separation during infancy affected fear learning and memory in adolescence and adulthood, but they did not investigate any neural changes to correlate with the behavior. An interesting and useful extension to the current study would be to examine the potential behavioral differences seen in fear conditioning and extinction behavior in adolescent offspring following this aversive caregiving paradigm.

4.4 Conclusion

Based on the evidence provided from this study, it is clear that the quality of maternal care experienced during infancy can produce global epigenetic modifications that extend beyond the period of maternal care into later development. Further research with this model and in humans will advance knowledge of the effects of early-life stress on gene regulation and behavioral outcomes. The link between these experiences and increased risk of developing mental illness is gaining attention in psychiatry and developmental psychology, which could help establish new guidelines

for child care and better therapeutic interventions for children who have experienced maltreatment.

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