CAREGIVER EXPERIENCES PRODUCE LASTING EPIGENETIC EFFECTS ON RAT HIPPOCAMPAL BDNF GENE ACTIVITY

by

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ABSTRACT

Current research in the fields of developmental psychology and neuroscience has revealed that there are sensitive periods during postnatal development in which the developing brain has a high level of plasticity, and that early-life caregiving experiences can shape neural circuits and determine the structural and functional aspects of brain and behavior into adulthood and even across generations. These early-life caregiver experiences produce epigenetic modifications, which are mechanisms that produce functional and heritable changes in the genome without altering the underlying DNA sequence. This is a gene-environment interaction that could influence risk or resiliency to later psychiatric disorders. This study focused on DNA methylation, an epigenetic mechanism that typically blocks and suppresses gene transcription. The stable nature of DNA methylation makes it ideal for determining long-lived gene effects that control brain function and behavior. In particular, this study assessed DNA methylation of the *Brain-derived neurotrophic factor* (*Bdnf*) gene, because it continues to be associated with long-term memory, the lasting effects of stress in adulthood, and neuropsychiatric disorders. Previous research with various rodent models suggests that early stress or variations in caregiving alter the expression of both *Bdnf* mRNA and BDNF protein levels in several regions, including the hippocampus. Regulatory mechanisms facilitating these early-life experience-induced

changes are not clear, thus I assessed *Bdnf* methylation and expression in the dorsal and ventral hippocampi of adult male and female rats with a history of adverse or nurturing caregiving. Biochemistry results demonstrate that exposure to adverse or nurturing caregiving experiences during the first postnatal week of life yield distinct patterns of *Bdnf* DNA methylation and gene expression patterns within the adult hippocampus. Furthermore, the specific nature of the patterns (i.e. increases or decreases) is dependent upon the animal's sex and *Bdnf* gene locus (exon I vs. exon IV).

Chapter 1

INTRODUCTION

1.1 Child Maltreatment and Animal Models

Child maltreatment, which can include physical abuse, sexual abuse, neglect, or emotional maltreatment (Cichetti & Toth, 1993), is a highly prevalent and major problem in our society today. Recently published U.S. government statistics estimate that there were 3.4 million referrals concerning the welfare of about 6.2 million children reported to Child Protective Services (CPS) agencies throughout the United States in 2011. Approximately 2 million of these reports were accepted for investigation, and 676,569 victims of child abuse and neglect were reported (Department of Human Health Services, 2012). Even worse, these statistics only reflect cases that were referred to CPS (and not all were investigated), so the actual number of abused and neglected children is likely to be much higher (Cichetti & Toth, 2005).

These findings are extremely troubling, considering that early postnatal life represents a period in which the quality of the infant's experience with their caregiver is associated with emotional and cognitive development (Fernald and Gunnar, 2009). Human studies suggest that nurturing early-life maternal care is associated with resilience to psychological disorders, while adverse early life conditions such as poverty, substance abuse by the mother, or maternal depression, have been associated with vulnerability to psychopathology later in life (Korosi & Baram, 2009).

Specifically, there is a significant association of reported childhood maltreatment and the later diagnosis of adolescent and adulthood schizophrenia, borderline personality disorder, anxiety disorders, posttraumatic stress disorder, major depression, as well as an increased risk of attempted suicide (Neigh, Gillespie, & Nemeroff, 2009; Repetti, Taylor, & Seeman, 2002; Cichetti & Toth, 2005; Loman & Gunnar, 2010, Dube et al., 2001). Furthermore, early life trauma can even have implications for somatic health, including increased risk for obesity (Gunstad et al., 2006), cardiovascular disease (Caspi, Harrington, Moffitt, Milne, & Poulton, 2006), and diabetes (Goodwin & Stein, 2004).

Research with nonhuman primates, the closest animal models of human child maltreatment, has likewise shown a strong link between early-life experiences and developmental outcomes. In addition to physical abuse, macaques also neglect their infants, and the behavioral changes in macaques replicate many of the findings seen in clinical studies with humans. Macaque mothers who exhibit high infant rejection, low responsiveness, and low protectiveness produce infants who grow up with signs of distress and irritability, such as high rates of tantrums and screams, and stunted social development, such as diminished exploration and play and delayed independence from mother (Sanchez, 2006).

Studies in rodents have also illustrated that early-life maternal care can lead to dramatic changes in brain structure and function and behavioral outcomes. Manipulations of the mother-pup relationship in the rat have been an important model in this area of research, and have demonstrated long-term consequences on behavior of the pups later in life. These include abnormalities such as deficits in information processing, impaired memory, heightened fear- and anxiety-like behaviors, altered

drug-seeking behavior, and social withdrawal (Gunnar & Quevedo, 2007; Meaney & Szyf, 2005; Korosi & Baram, 2009; Sanchez, 2006).

1.2 Stress and HPA Axis development

During early development, the brain has a high level of plasticity that allows environmental signals to modify the trajectories of neural circuits. This can shape stress-regulating pathways that underlie emotional functions and endocrine responses to stress, which can lead to long-lasting altered stress responsivity during adulthood (Murgatroyd & Spengler, 2011). Imaging studies on adults who report child maltreatment display a number of lasting neural abnormalities (De Bellis, 2005; Gunnar & Quevedo, 2007), suggesting that abnormal function and responsiveness of the prefrontal cortex, hippocampus, amygdala, and other brain regions involved in the hypothalamic-pituitary-adrenal (HPA) axis, a major part of the neuroendocrine system that controls reactions to stress, are likely contribute to the cognitive dysfunction associated with childhood maltreatment.

When exposed to acute stress, the stress response is extremely adaptive because it shifts biological resources toward physiological functions that promote escape and survival (McEwen, 2008). Adaptive responses to stress are characterized by a relatively rapid increase of cortisol, the end hormone product of the HPA axis, and followed by a progressive decline. However, if the stress response becomes chronic due to repeated exposure to stressors, and cortisol is excreted excessively, then this results in persisting hyperreactivity of the physiological response to stress, increasing the risk of stress-related disease such as depression (Heim, Mletzko,

Purselle, Musselman, & Nemeroff, 2008). Accumulating evidence also suggests that HPA hyperreactivity early in life leads to blunted HPA axis reactivity in adulthood (Tyrka et al., 2008), and this has been shown in animal models as well. Variation in parenting style occurs naturally in macaques, and those with higher rates of rejection in their first month of life had elevated plasma cortisol levels when compared to non-abused infants. By 6 months of age, the macaques exhibited blunted basal cortisol levels compared to controls (McCormack, Sanchez, Bardi, & Maestripieri, 2006).

One area of the brain that is particularly vulnerable to high levels of stress is the hippocampus. This is due to the fact that it has a high level of glucocorticoid receptors, to which cortisol and other glucocorticoids bind, and is heavily involved in glucocorticoid-mediated negative feedback on HPA axis activity. In clinical studies, early-life stress has been shown to be associated with decreased hippocampal volume (Driessen et al., 2000, Vythilingam et al., 2002), and in animal studies early life stress has also been shown to lead to the inhibition of neurogenesis (Karten, Olariu, & Cameron, 2005) and abnormalities in dendritic remodeling (Vyas, Mitra, Shankaranarayana Rao, & Chattarji, 2002) and long-term potentiation (Pavlides, Nivon, & McEwen, 2002). Implications of these effects could likely include dysregulation of the HPA axis and cognitive impairment.

1.3 Epigenetic Mechanisms as Potential Mediators of Gene-Environment Interactions

Recently, investigators have begun to explore epigenetic modifications, changes in genes that do not alter the DNA sequence, as potential mechanisms for how

early experiences produce individual differences in neural function and behavior. Since events occurring during postnatal development have effects that can persist into adulthood, epigenetic mechanisms are proposed to respond dynamically to these environmental factors, enabling both immediate and possibly sustained changes in gene expression. A proposed model for this is that initially, an environmental cue activates signaling pathways that lead to the binding (or dissociation) of certain transcription factors at specific regulatory sequences within the DNA sequence, which produces changes in chromatin structure (i.e. DNA and its associated histone proteins in the nucleus). This initial signal is enough to alter gene expression programmed within the cell, but for these changes to be long-lasting it must also trigger another epigenetic mechanism that will serve to maintain the remodeled chromatin in the absence of more environmental cues (Berger, Kouzarides, Shickhattar, & Shilatifard, 2009).

Variations in these gene-environment interactions might allow us to explain how some organisms are able to resist the effects of stress and display resilience, whereas others are vulnerable and succumb to its effects (Dudley, Li, Kobor, Kippin, & Bredy, 2011; Champagne, 2010). Recent studies suggest that dynamic alterations in epigenetic modifications are crucial for the synaptic remodeling that mediates learning and memory (Bredy et al., 2007; Lubin, Roth, & Sweatt, 2008; Tsankova et al., 2006). If epigenetic regulation of genes plays an active process in regulating an animal's ability to respond to and form memories of its environment and experiences, then epigenetic modifications of genes in early development could certainly have the capacity to subsequently affect cognition. Indeed, DNA methylation and histone acetylation, another type of epigenetic modification, have now been linked to

adulthood learning and memory, drug addiction, and several psychiatric and neurological illnesses (Franklin & Mansuy, 2010; LaPlant & Nestler, 2010; Roth, Lubin, Sodhi, & Kleinman, 2009).

DNA methylation is the most commonly studied epigenetic modification with regards to experience-induced changes in the postnatal environment, and has a stable and even heritable nature regarding gene regulation that offers it as an ideal substrate for long-lasting cellular alterations. In the cell nucleus, DNA is wrapped around a core of structural proteins called histones, and this complex forms the chromatin. One can think of it like thread wrapped around a spool, and this complex loosens or tightens based on the type of epigenetic modification, which in this case DNA methylation most often tightens. The interaction between histones and DNA is partly mediated by the N-terminal tail of histone proteins, and covalent modifications to these tails help to control whether DNA can be accessed for gene transcription (Strahl & Allis, 2000). When DNA is methylated, methyl groups are added to cytosines, which occur at cytosine-guanine (CG) dinucleotides. These sequences can be found primarily within the promoter region of a gene and thus are in a good place to control the level of expression of a gene. DNA methylation is catalyzed by DNA methyltransferases (DNMTs). Methyl CpG binding protein 2 (MeCP2) binds to methylated cytosines, and recruits histone deacetylases (HDACs) and other co-repressors (Bird, 2002). This promotes higher-affinity interaction between DNA and histone core, condensing the chromatin, and typically suppresses transcription factor binding and gene expression. DNA methylation was once considered both unique to embryonic development and irreversible, but now there is a growing body of evidence that the state of DNA methylation at specific genomic sites is indeed dynamic, continues throughout the

lifespan, and can even be transmitted across generations (Radtke et al, 2011; Roth et al, 2009; Franklin et al, 2010).

1.4 Human Epigenetic Research and Animal Models

Although epigenetic research with humans is limited with using post mortem and peripheral tissue samples, it does provide useful insight into the role of DNA methylation in predisposing individuals to psychopathology. For example, it has been demonstrated that DNA methylation of the exon 1F promoter of the *glucocorticoid receptor* (*GR*) gene in hippocampal tissue was increased in suicide victims compared to controls if suicide was accompanied with developmental history of childhood maltreatment (McGowan et al., 2009). Further, analysis of cortical tissue samples from bipolar and schizophrenic patients indicates a general hypermethylation compared with individuals having no history of mental illness (Veldic, Guidotti, Maloku, Davis, & Costa, 2005). Specifically, studies have consistently found reductions in expression of *Reelin* and *GAD1* genes, which support synaptic function and memory, in the hippocampus and cortex of both male and female schizophrenic patients (Costa et al., 2009; Grayson et al., 2009).

Other evidence of early epigenetic marking in humans comes from studies examining whether there is epigenetic fetal programming by environmental influences. For example, male and female infants of mothers with high levels of depression and anxiety during the third trimester have increased methylation of the *Nr3c1* (*GR*) gene promoter in cord blood cells. Moreover, the methylation of the

neonatal *GR* promoter predicted increased salivary cortisol levels of infants at 3 months of age (Oberlander et al., 2008).

One concern with human studies is that causality between early-postnatal experience and long-term outcomes is difficult to infer because their genetic background cannot be controlled. Therefore, it is important to specifically address the mechanisms by which gene-environment interactions can predispose individuals toward later psychopathology by developing animal models in which early environmental factors can be manipulated in a controlled manner (Murgatroyd & Spengler, 2011). With animals, one can control for genetic background and other parameters of interest, as well as directly access specific brain regions for invasive neuroanatomical, biochemical and genetic approaches (Korosi & Baram, 2009).

In maternally and socially isolated infant macaques, increased stress has been linked to hypermethylation of the serotonin transporter gene, in which its changes have been linked to a number of psychological disorders (Kinnally et al., 2010). Differential rearing (either maternal or surrogate-peer rearing) of macaques is also associated with differential DNA methylation in both prefrontal cortex and immune cells in early adulthood (Provencal et al., 2012). Michael Meaney and his colleagues have extensively studied the differences in offspring of rats, which engage in high and low levels of maternal care (Meaney & Szyf, 2005; Szyf, Weaver, & Meaney, 2007; Zhang, Parent, Weaver, & Meaney, 2004; Weaver et al., 2004; Szyf et al., 2005) and these studies have linked the differences in HPA axis responsiveness to an epigenetic change that is catalyzed by the varying levels of maternal care. Specifically, rat mothers that demonstrated high levels of nurturing care (increased pup licking and grooming and arched-back nursing) had offspring with less DNA methylation of the

GR gene promoter in their hippocampus compared to offspring who had mothers with low levels of nurturing behavior (Weaver et al., 2004). They also had higher *GR* expression. There were also alterations in histone acetylation and transcription factor (NGFI-A) binding to the *GR* promoter between offspring of different care types. The increases in NGFI-A induced by higher levels of maternal care are hypothesized to increase transcription and thereby increase glucocorticoid receptor mRNA (Szyf et al., 2005).

1.5 The *Brain-Derived Neurotrophic Factor* (*Bdnf*) Gene

The BDNF protein has an important role in normal neural development. It is highly expressed in limbic structures and cerebral cortex, and is important for long-term potentiation and neurogenesis, making it important for learning and memory as well as reward-related processes. Past research has indicated that BDNF could be an important factor for determining risk or resiliency to the onset of various psychological disorders. It has been found that chronic stress exposure and associated negative emotional responses are generally linked to decreased levels of BDNF protein. For example, exposure to stress and the stress hormone corticosterone have been shown to decrease the expression of BDNF in rats (Uys et al., 2006), and lead to an eventual atrophy of the hippocampus if exposure is persistent. Atrophy of the hippocampus and other limbic structures has been shown to take place in humans suffering from chronic depression (Warner-Schmidt & Duman, 2006).

The *Bdnf* gene, which codes for the BDNF protein, has a complex structure displaying differential exon regulation and usage, suggesting a subcellular- and brain

region-specific distribution as well. A number of studies have also presented substantial similarities in rodents and humans (Liu et al., 2006, Liu et al., 2005). This demonstrates that the structure is homologous across species, indicating a high degree of translatability. Recent interest has been directed to the epigenetic regulation of *Bdnf* gene that mediates the effects of environmental factors on enduring changes of its expression. In both humans and animal models, since the *Bdnf* gene's neurotrophic actions play an important role in early brain development and neuronal plasticity, and because BDNF exhibits activity-regulated release in the CNS (Greenberg et al., 2009; Hennigan et al., 2007), abnormal regulation of this gene is a leading candidate molecular mechanism through which early-life adverse experiences are able to produce stable neurobiological modifications (Branchi, Francia, & Alleva, 2004; Fumagalli et al., 2007; Casey et al., 2009).

1.5 Rationale for the Current Study

Previous work from Tania Roth has provided novel evidence regarding an epigenetic basis for lasting effects of early-life experiences on the *Bdnf* gene (Roth et al., 2009). It was demonstrated that maltreatment of infant rats by caregivers during the first seven days of life produced increased methylation of the *Bdnf* gene in the prefrontal cortex of both male and female rats, an effect also associated with decreased *Bdnf* mRNA levels in adulthood (3 months after maltreatment). Sequencing of an important regulatory region of the *Bdnf* gene (DNA associated with exon IV) revealed that across 12 CG dinucleotide sites (sites that can be methylated) within that regulatory region, adults with a normal infancy had either no or very little cytosine

methylation. Conversely, in the adults who had experienced the adverse conditions during infancy those same CG sites were all highly methylated.

Previous research indicates that the dorsal (DH) and ventral (VH) hippocampi are wired independently in a way that allows for different functional capabilities (Maggio & Segal 2009, Hawley & Leasure 2012). It is also well accepted that the fundamental organization of hippocampal connectivity is very consistent in rats, monkeys, and humans, suggesting similar roles across species (Faneslow & Dong 2010). It is clear that the DH is primarily involved in the cognitive process associated with learning and memory (including navigation, exploration, and locomotion), whereas the VH is associated with motivational and emotional behavior. Although there has been past research assessing epigenetic regulation of the *Bdnf* gene within the DH and VH in a PTSD rat model (Roth, Zoladz, Sweatt, & Diamond, 2011), regulatory mechanisms facilitating early-life experience-induced changes in *Bdnf* activity within these brain areas is not known. To better understand mechanisms responsive to early-life experiences, *Bdnf* DNA methylation and gene expression of these structures were examined in adult rats exposed to nurturing or adverse care during infancy.

In this study, I used a limited bedding regimen (Roth et al., 2009) to generate early-life stress by a present, stressed mother instead of using infant-caregiver separation in an attempt to better model an abusive/neglectful experience in humans. A hallmark of maternal behavior in neglect/abuse situations is its unpredictability and fragmented quality (Ivy et al., 2008). A limited bedding regimen has been used in other laboratories to produce stressful early-life environmental conditions that evoke changes in later behavior (less licking/grooming, fragmented interactions with the

pups, and increased levels of the stress hormone corticosterone) (Ivy et al., 2008; Roth and Sullivan, 2005). Further, since the first week of life in the rat is crucial for appropriate maturation of the stress response and the underlying hypothalamic-pituitary-adrenal (HPA) axis, and it a period of intense brain growth (Plotsky & Meaney, 1993), this is the time period in which we exposed infant rats to nurturing or adverse caregiving conditions.

The current study investigates the hypotheses that: 1) exposure to nurturing or adverse caregiving conditions produces distinct epigenetic markings of *Bdnf* DNA that are present within the adult DH and VH; and, 2) these epigenetic patterns will correlate with patterns of *Bdnf* gene expression. Since the outcomes of these early-life manipulations likely vary between sexes, as well as across various *Bdnf* gene loci, I also explored patterns in both males and females and at multiple gene loci.

Chapter 2

METHODS

2.1 Subjects

Male and female outbred Long-Evans rats were obtained from Harlan and housed in our breeding colony. Animals were housed in polypropylene cages with plentiful wood shavings in a temperature and light-controlled colony room (12-hours light/dark cycle with lights on at 6:00am) with *ad libitum* access to food and water. All behavioral manipulations were performed during the light cycle. All females had experience in raising at least one litter prior to the beginning of the experiment so that no first-time mothers were ever used. Postnatal day (PN) 0 was designated as the day of pup birth, and on PN1, litters were culled to 6 males and 6 females. 17 litters were used to generate PN90 cohorts for tissue collection. The University of Delaware Animal Care and Use Committee approved all procedures.

2.2 Caregiving Manipulations

With a method previously used (Roth et al., 2009) and adapted from earlier studies (Ivy et al., 2008; Roth & Sullivan, 2005), infant rats were divided into three equal groups on PN1 using a within litter design. For thirty minutes a day beginning on PN1 and ending on PN7, up to 2 male and 2 females pups from the same litter were

exposed to one of three conditions. In the maltreatment condition, the caregiver was a lactating, non-biological female who was placed in a novel environment with inadequate nesting material (only 100 ml of wood shavings) in order to facilitate abusive behaviors towards the pups. In the cross-foster care condition, the caregiver was another lactating, non-biological female who was given at least one hour to habituate to the exposure chamber and provided ample wood shavings (an approximate 2cm layer across the chamber floor) for nesting material in order to facilitate nurturing behaviors. After the 30 minute session, experimental pups were removed from the test chamber and placed back into the homecage with the biological mother. Stimulus dams (maltreatment and cross-foster) were also reunited with their biological pups immediately after each exposure session. In the normal maternal care condition, remaining pups from the litter were only marked for identification and weighed, and returned to the biological mother in the homecage to serve as a control group. Chamber temperatures for the maltreatment and cross-foster care conditions were maintained between 27-30 °C to help maintain pup body temperature. Except for weekly cage changes, pups remained undisturbed until PN21-23 when they were housed in same-sex pairs through adolescence and into adulthood. Nurturing and abusive behaviors were scored for all 3 conditions via video recordings, and 40 kHz ultrasonic (Batbox III D, NHBS Ltd., UK) and audible vocalizations from infant rats during each exposure session were scored from digital recordings. 40 kHz ultrasonic vocalizations were used because these are generally emitted in response to distressing situations, such as prolonged separation from the mother (Hofer, 1996). For caregiver behavior observations, adverse or nurturing behaviors were tallied in five minute time bends and averaged across the 7 exposure days. Vocalizations were coded by marking

if a vocalization was heard or not for each minute time bend during the thirty minute session and then averaged across the 7 exposure days as well.

2.3 DNA Methylation and Gene Expression Assays

Animals were sacrificed at baseline conditions at PN90 and female estrous cycles were measured by post-mortem vaginal lavage. Brains were removed, sliced using a 1 mm brain matrix, flash frozen on untreated slides with 2-methylbutane, and placed in a -80 °C freezer until later processing. The hippocampus (dorsal vs. ventral tissue) was dissected on dry ice using stereotaxic coordinates, and DNA/RNA were simultaneously extracted (Qiagen Inc., Valencia, CA). Quantification and assessment of nucleic acid quality from samples were determined using spectrophotometry (NanoDrop 2000). Methylation status was later assessed via direct bisulfite DNA sequencing (BSP, on a Bio-Rad CFX96 system) on bisulfite-modified DNA (Qiagen Inc., Valencia, CA). I used BSP because it is the more quantitative method compared to methylation specific real-time PCR (MSP), as it assesses methylation status of individual CG sites, and has been shown to be more reliable (Shiraz et al., 2012). Bisulfite-treated samples were amplified by primer sets targeting DNA associated with *Bdnf* exons I and IV, because these have been shown to be important regulatory sites of this gene and to be epigenetically regulated (Bredy et al., 2007; Tsankova et al., 2006; Lubin et al., 2008). PCR products were purified and sequenced at the Delaware Biotechnology Institute (http://www.dbi.udel.edu/core/dnasequencing). To confirm that direct bisulfite sequencing was adequately sensitive to detect methylation, universally unmethylated and methylated standards (EpigenDx) were run and analyzed

in parallel to the samples. Reverse transcription was performed using a cDNA synthesis kit (Qiagen) on RNA, and cDNA was amplified by real-time PCR (Bio-Rad CFX96) with Taqman probes (Applied Biosystems) to target all *Bdnf* transcripts (thus aimed at detecting exon IX sequences) or *tubulin* (for a reference gene) mRNA. All reactions for each gene in gene expression assays were run in triplicate. Product specificity for BSP and gene expression was verified by electrophoresis on a 2% agarose gel.

2.4 Statistical Analysis

Differences were analyzed by ANOVA, one-sample t-tests, two-tailed unpaired t-tests, and Bonferroni post-hoc tests where appropriate. For all analyses, significance was set at $p \leq 0.05$ unless otherwise specified.

Chapter 3

RESULTS

3.1 Caregiver Behaviors

To manipulate the quality of early-life caregiving experiences, male and female rat pups were exposed to maltreatment or nurturing care either with a non-biological mother outside of the homecage (cross-foster care) or within their homecage with their biological mother (normal care). This study used part of a larger cohort in which it was found that caregiving conditions differed greatly across our treatment groups, as there was a main effect of caregiving behavior ($F_{1,98}$=245.8, $p<0.001$) and a behavior x infant condition interaction ($F_{2,98}$=173.7, $p<0.001$). Figure 1a shows that infants exposed to normal care or cross-foster care experienced high levels of nurturing care and low levels of adversity, which did not differ between the two conditions ($p>0.05$). In contrast, infants exposed to maltreatment experienced a significant amount of adverse treatment and less nurturing care ($p<0.001$). The types and percentages of infant-directed behaviors within the normal and cross-foster care conditions are illustrated in Figure 1b-c, and consisted primarily of licking, grooming, nesting, and nursing. These behaviors were less prevalent within the maltreatment condition, and instead dams predominately stepped on, dropped during transport, dragged while nipple attached, actively avoided, and roughly handled infants (Figure

1d). Analyses for each type of infant-directed behavior between the 3 conditions revealed no differences in levels of individual behaviors between the normal and cross-foster care conditions but significant differences when compared to the maltreatment condition (Table 1).

	One-way ANOVAs			*p-values for Bonferroni's tests*		
	F	*df*	*p value*	*CFC vs. NMC*	*MAL vs. CFC*	*MAL vs. NMC*
Step on	21.39	51	<0.001	>0.05	<0.001	<0.001
Drop	14.99	51	<0.001	>0.05	<0.001	<0.001
Drag	2.29	51	0.112	n/a	n/a	n/a
Actively Avoid	36.61	51	<0.001	>0.05	<0.001	<0.001
Roughly Handle	27.04	51	<0.001	>0.05	<0.001	<0.001
Lick/Groom	14.94	51	<0.001	>0.05	<0.001	<0.01
Crouch/Nurse	19.92	51	<0.001	>0.05	<0.001	<0.001

Table 1. Analyses of individual infant-directed behaviors across the three conditions. CFC=cross-foster care; NMC=normal care; MAL=maltreatment (Blaze et al., in press).

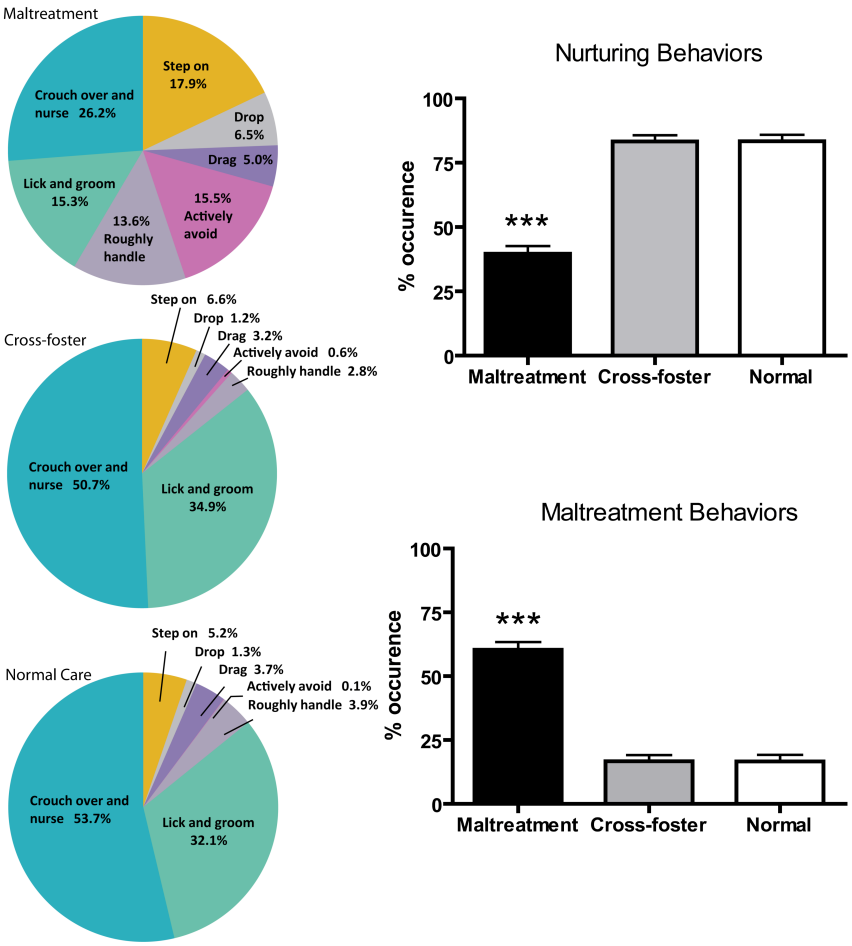

Figure 1. Differences in caregiving behaviors across treatment groups. To manipulate the early-life caregiving environment, infant male and female rats experienced nurturing care within the homecage (normal care), were exposed to nurturing care outside the homecage (cross-foster), or were exposed to caregiver maltreatment outside the homecage. A) Behavioral observations show that there were significant differences in the amount of nurturing and adverse caregiving behaviors displayed by dams across the three treatment groups. Assessment of individual behaviors experienced by infants across the three conditions revealed high levels of infant licking, grooming, and nursing in the (B) normal and (C) crossfoster care conditions but aberrant caregiving behaviors in the (D) maltreatment condition. n=18-22 dams/group; error bars=SEM (Blaze et al., in press).

3.2 Infant responses to caregiving conditions

To determine infant responses to our caregiving environments, we measured audible and ultrasonic (40 kHz) vocalizations emitted during each exposure session in each of the 3 conditions (Figure 2). Once again this study used part of a larger cohort in which both audible ($F_{2,40}$=7.29, $p<0.01$) and ultrasonic ($F_{2,20}$=110.5, $p<0.001$) vocalizations differed significantly across our treatment groups. While there was no difference in audible or ultrasonic vocalizations emitted between infants in the normal and cross-foster care conditions ($p>0.05$), infants in the maltreatment condition emitted significantly more audible ($p<0.05$ vs. normal and $p<0.01$ vs. cross-foster care) and ultrasonic ($p<0.001$ vs. normal and cross-foster care) vocalizations.

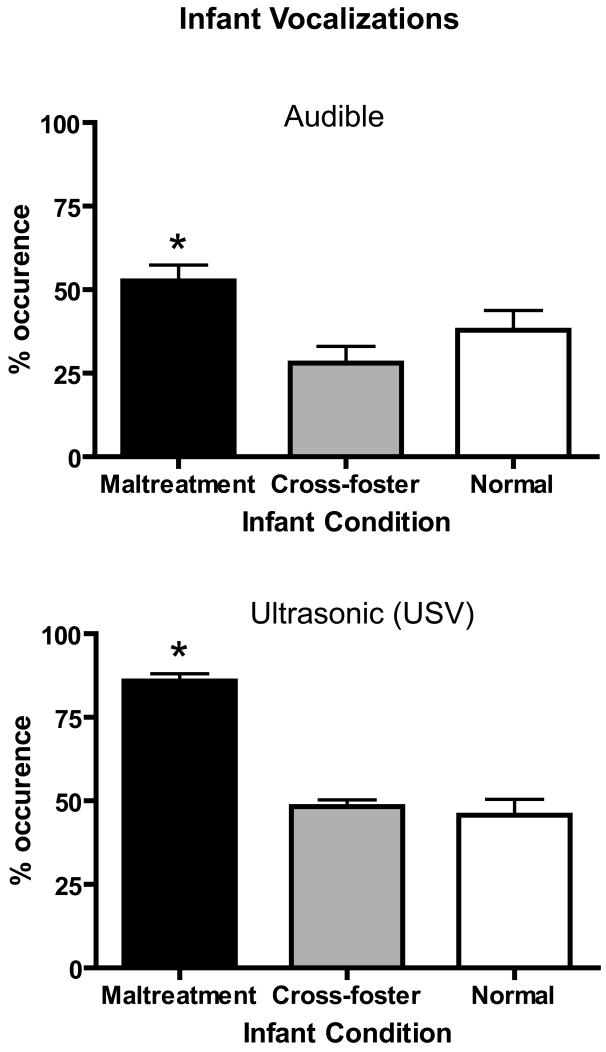

Figure 2. Pup responses to caregiving environments. Infants in the maltreatment condition emitted significantly more (A) audible and (B) ultrasonic vocalizations compared to infants in the normal and cross-foster care conditions. **p<0.01, ***p<0.001; error bars=SEM (Blaze et al., in press).

3.3 DNA Methylation Patterns

DNA methylation of the *Bdnf* gene was measured 3 months (PN90) after our caregiving manipulations. Specifically, BSP was used to characterize methylation of

DNA associated with *Bdnf* exons I and IV. Since methylation patterns did not vary substantially across estrus stages in females, female data were collapsed. A two-way ANOVA revealed that in the female VH, there was a main effect of infant condition ($F_{2,290}$=22.28, p<0.001) and CG site ($F_{9,290}$=3.590, p<0.001) for *Bdnf* exon I (Figure 3a). In the male VH, there was also a main effect of CG site ($F_{9,300}$=30.47, p<0.001) for *Bdnf* exon I (Figure 3b). A one-way ANOVA revealed that the means were significantly different across treatments for females when the CG variable was collapsed ($F_{2,317}$=21.24, p<0.001). Using Bonferroni's post-hoc tests, it was found that maltreated females had significantly higher levels of methylated DNA at *Bdnf* exon I compared to cross-foster care (t=5.45, p<0.001) and normal care controls (t=5.78, p<0.001) when collapsed across CG sites (Figure 3c). There were no significant differences in levels of methylated DNA at *Bdnf* exon I in the male VH when collapsed across CG sites (all p's>0.05) (Figure 3d).

A two-way ANOVA revealed that in the female DH, there was a main effect of CG site ($F_{9,300}$=64.66, p<0.001) for *Bdnf* exon I (Figure 4a). In the male DH, there was also a main effect of CG site ($F_{9,268}$=23.22, p<0.001) and infant condition ($F_{2,268}$=5.55, p<0.01) for *Bdnf* exon I (Figure 4b). A one-way ANOVA revealed that the means were significantly different across treatments for males collapsed across CG sites ($F_{2,295}$=3.13, p<0.05) Figure 4d) . There were no significant differences in the levels of methylated DNA at *Bdnf* exon I in females in the DH when collapsed across CG sites (p>0.05) (Figure 4c).

A two-way ANOVA revealed that in the female VH, there was a main effect of infant condition ($F_{2,359}$=7.72, p<0.001) and CG site ($F_{11,359}$=9.61, p<0.001)for *Bdnf* exon IV (Figure 5a). In the male VH, there was also a main effect of CG site

($F_{11,336}$=12.38, p<0.001) and infant condition ($F_{2,336}$=20.54, p<0.001) for *Bdnf* exon IV (Figure 5b). A one-way ANOVA revealed that the means were significantly different across treatments for both females ($F_{2,392}$=6.57, p<0.01) and males ($F_{2,369}$=15.18, p<0.001) collapsed across CG sites. Using Bonferroni's post-hoc tests, it was found that maltreated males had significantly higher levels of methylated DNA across *Bdnf* exon IV compared to cross-foster care (t=3.21, p<0.01) and normal care controls (t=5.34, p<0.001) (Figure 5d). Further, cross-foster care females had significantly higher levels of methylated DNA at *Bdnf* exon IV compared to normal care controls when collapsed across CG sites (t=3.61, p<0.01) (Figure 5c).

A two-way ANOVA revealed that in the female DH, there was a main effect of infant condition ($F_{2,384}$=3.4, p<0.05) and CG site ($F_{11,384}$=16.75, p<0.001) for *Bdnf* exon IV (Figure 6a). In the male DH, there was also a main effect of CG site ($F_{11,312}$=3.84, p<0.001) and infant condition ($F_{2,312}$=25.61, p<0.001) for *Bdnf* exon IV (Figure 6b). A one-way ANOVA revealed that the means were significantly different across treatments for males collapsed across CG sites ($F_{2,345}$=23.93, p<0.001), but not for females (p>0.05). Using Bonferroni's post-hoc tests, it was found that normal-care males had significantly higher levels of methylated DNA at *Bdnf* exon IV compared to cross-foster care (t=6.08, p<0.001) and maltreated (t=5.64, p<0.001) rats when collapsed across CG sites (Figure 6d).

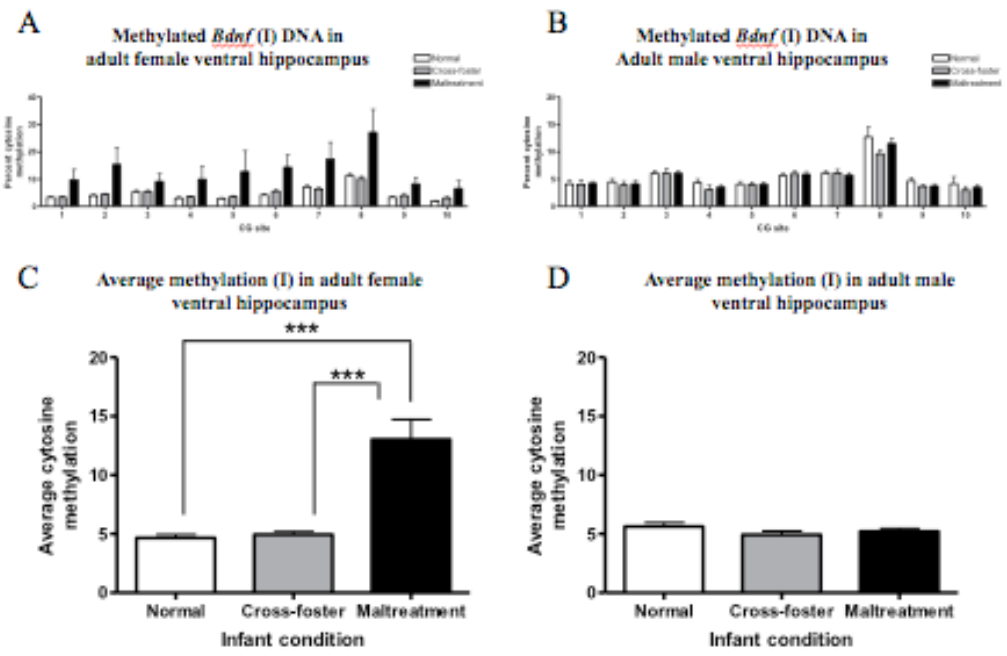

Figure 3. Methylated *Bdnf* (I) DNA and average methylation in adult female and male ventral hippocampus. (A) BSP results indicate that in the female ventral hippocampus, there was a main effect of infant condition and CG site for *Bdnf* exon I. (B) In the male ventral hippocampus, there was also a main effect of CG site for *Bdnf* exon I. When collapsed across CG sites (C), maltreated females had significantly higher levels of methylated DNA at *Bdnf* exon I compared to cross-foster care and normal care controls. (D) There were no significant differences in levels of methylated DNA at *Bdnf* exon I in the male ventral hippocampus when collapsed across CG sites.**$p<0.01$, ***$p<0.001$; Males, n=7-14/group; females, n=10-12/group; subjects derived from 17 litters; error bars=SEM.

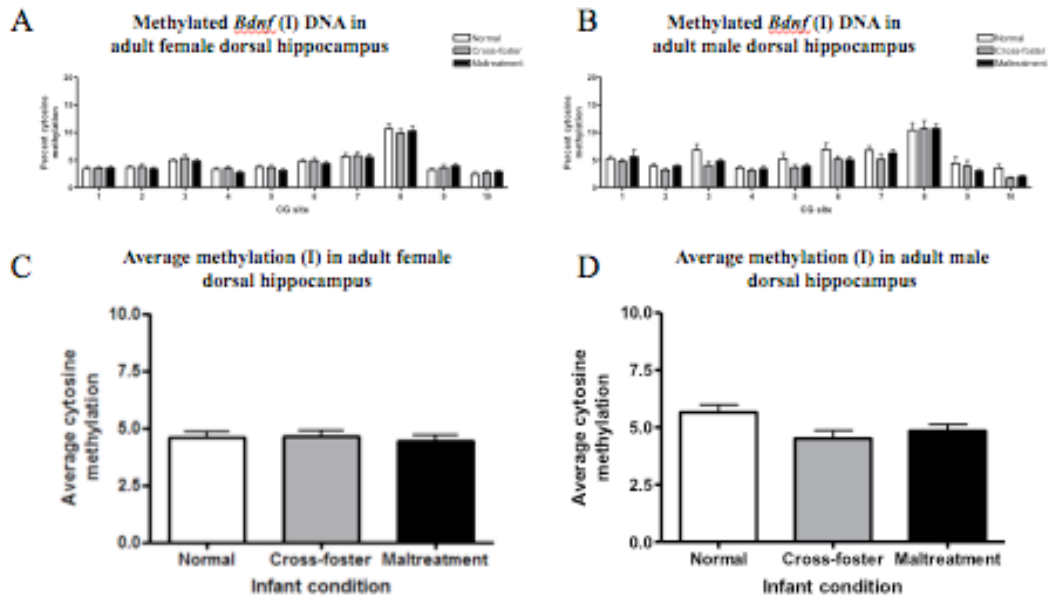

Figure 4. Methylated *Bdnf* (I) DNA and average methylation in adult female and male dorsal hippocampus. (A) BSP results indicate that in the female dorsal hippocampus, there was a main effect of CG site for *Bdnf* exon I. (B) In the male dorsal hippocampus, there was also a main effect of CG site and infant condition for *Bdnf* exon I. When collapsed across CG sites (C), the means were not significantly different across treatments for females but (D) were significant for males. **p<0.01, ***p<0.001; Males, n=7-14/group; females, n=10-12/group; subjects derived from 17 litters; error bars=SEM.

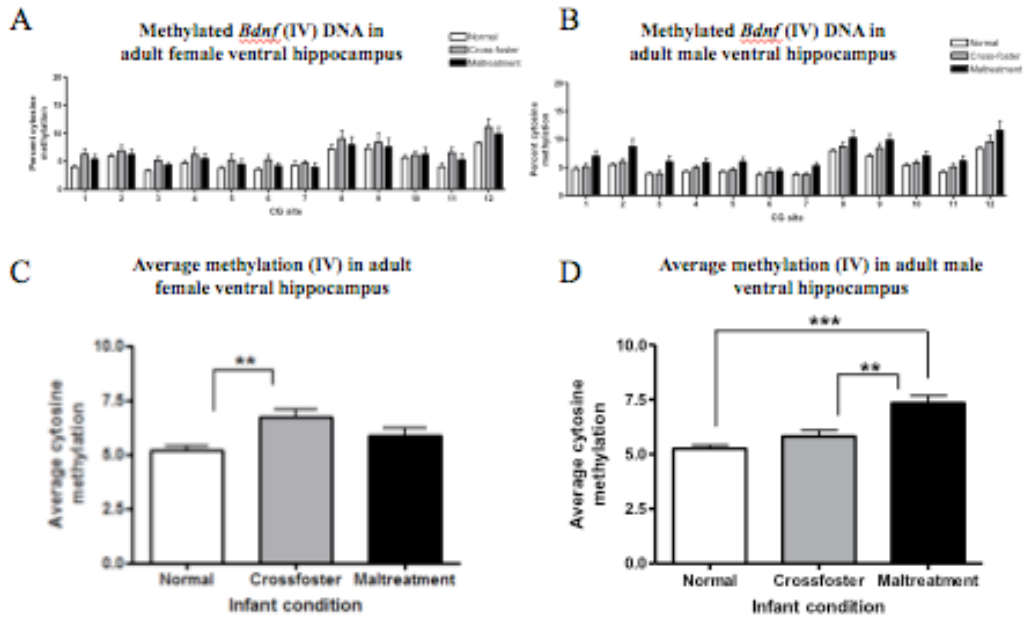

Figure 5. Methylated *Bdnf* (IV) DNA and average methylation in adult female and male ventral hippocampus. (A) BSP results indicate that in the female ventral hippocampus, there was a main effect of infant condition and CG site for *Bdnf* exon IV. (B) In the male ventral hippocampus, there was also a main effect of CG site and infant condition for *Bdnf* exon IV. When collapsed across CG sites (C), cross-foster care females had significantly higher levels of methylated DNA at *Bdnf* exon IV compared to normal care controls, and (D) maltreated males had significantly higher levels of methylated DNA at *Bdnf* exon IV compared to cross-foster care and normal care controls.**p<0.01, ***p<0.001; Males, n=7-14/group; females, n=10-12/group; subjects derived from 17 litters; error bars=SEM.

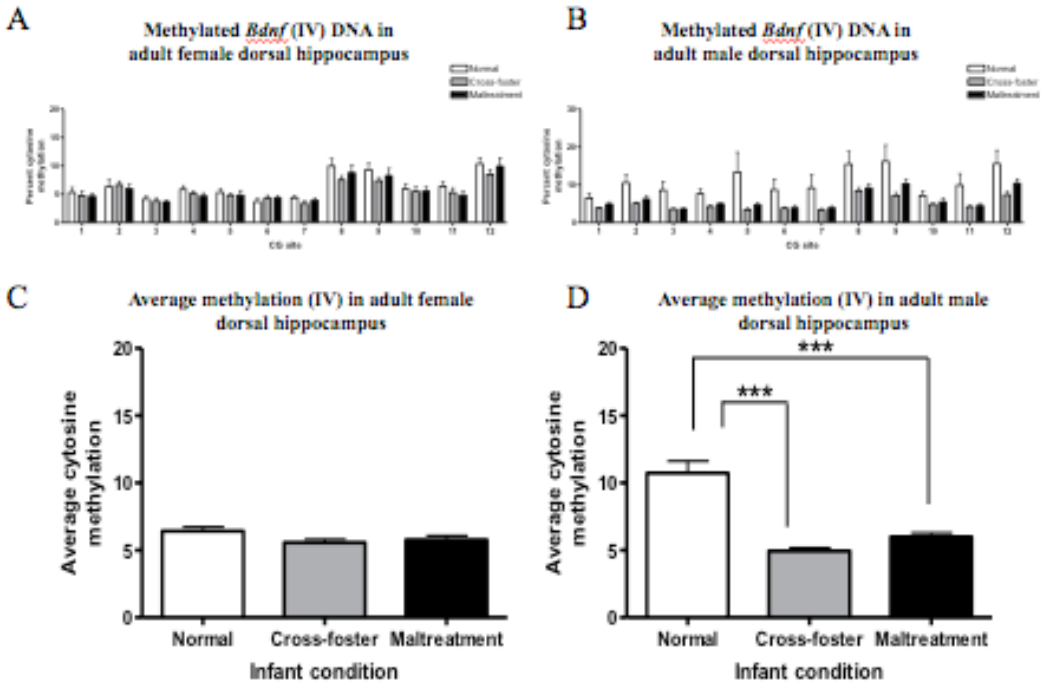

Figure 6. Methylated *Bdnf* (IV) DNA and average methylation in adult female and male dorsal hippocampus. (A) BSP results indicate that in the female dorsal hippocampus, there was a main effect of infant condition and CG site for *Bdnf* exon IV. (B) In the male dorsal hippocampus, there was also a main effect of infant condition and CG site for *Bdnf* exon IV. When collapsed across CG sites (C), there were no significant differences in levels of methylated DNA at *Bdnf* exon IV in the female dorsal hippocampus, but (D) in the male dorsal hippocampus, normal care males had significantly higher levels of methylated DNA at *Bdnf* exon IV compared to cross-foster care and maltreated rats.**$p<0.01$, ***$p<0.001$; Males, n=7-14/group; females, n=10-12/group; subjects derived from 17 litters; error bars=SEM.

3.4 Gene Expression

To examine whether *Bdnf* DNA methylation changes in adult rats coincided with alterations in *Bdnf* gene expression at baseline conditions, all *Bdnf* mRNA transcripts (exon IX-containing) were measured in tissue samples. In the VH, one-sample t-tests revealed that neither maltreated- or cross-fostered females differed significantly from normal care controls in gene expression (all p's>0.05). A two-way ANOVA also revealed a significant sex x infant condition interaction ($F_{1,38}$=5.23, p<0.05). A two-tailed unpaired t-test revealed that maltreated females had significantly lower levels of *Bdnf* gene expression in comparison to cross-foster care females (t=2.29, p<0.05) (Figure 7a).

In the DH**,** one-sample t-tests comparing maltreated or cross-foster care groups to normal care controls revealed that maltreated females had significantly lower levels of *Bdnf* gene expression compared to controls (t=2.93, p<0.05) (Figure 7b). A two-way ANOVA revealed a marginal sex x infant condition interaction ($F_{1,40}$=3.523, p=0.068), and a two-tailed unpaired t-test revealed that maltreated males had significantly higher levels of *Bdnf* gene expression compared to maltreated females (t=2.23, p<0.05) (Figure 7b).

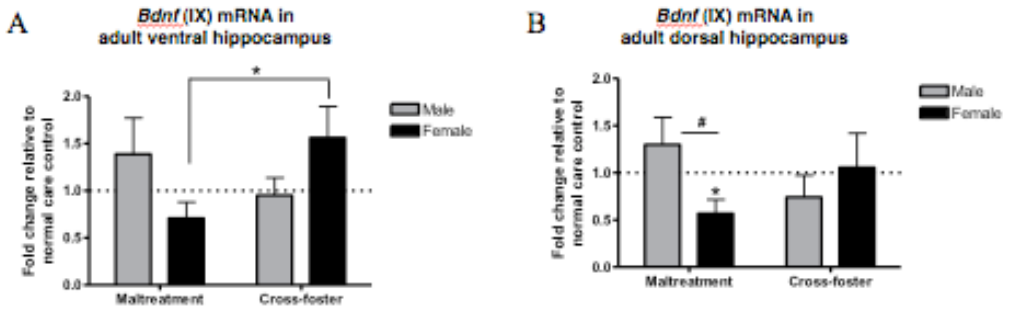

Figure 7. *Bdnf* (IX) mRNA in adult ventral and dorsal hippocampus. (A) In the ventral hippocampus, maltreated females had significantly lower levels of *Bdnf* gene expression compared to cross-foster care females. (B) In the dorsal hippocampus, maltreated females had lower levels of *Bdnf* gene expression compared to controls, and maltreated males had significantly higher levels of *Bdnf* gene expression compared to maltreated females. **p<0.01, ***p<0.001; Males, n=7-14/group; females, n=10-12/group; subjects derived from 17 litters; error bars=SEM.

Chapter 4

DISCUSSION

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

The current study was designed to investigate a link between caregiving conditions in infancy and *Bdnf* DNA methylation patterns within the dorsal and ventral hippocampus in adulthood. This was assessed in both male and female adult (PN90) rats using a model where infants were assigned within the same litter to different, reoccurring treatments. Infant rats were exposed to adversity outside of the homecage, which provides a unique way to study infant-caregiver dynamics without confounds such as the metabolic consequences of maternal milk or warmth deprivation. An additional strength of this model is the cross-foster and normal care control groups, which allow for discrimination of effects produced by exposure to another caregiver/caregiving environment and removal from the home cage/biological mother from those produced by caregiver maltreatment.

To produce maltreatment behaviors towards infant rats, a lactating female's nesting material was restricted while she was in a novel environment, and this produced high levels of aversive caregiving behaviors towards pups. It was observed that both lactating females in a familiar environment with adequate nesting material (cross-foster care condition) and the biological caregivers within their homecage

(normal care condition) exhibited similar high levels of nurturing care. It was also observed that infants responded differentially to the three caregiving conditions, with high amounts of audible and ultrasonic distress vocalizations emitted within the adverse caregiving environment but not within the environments where pups experienced nurturing care (normal and cross-foster care).

These data replicate those from previous studies from our lab and complement others demonstrating that resource deprivation within the homecage has the ability to produce adverse caregiving behaviors (Blaze et al., in press; Roth et al., 2009; Roth & Sullivan 2005; Ivy et al., 2008).

4.2 DNA Methylation Patterns are Influenced by Early-Life Caregiving Experiences

Biochemical findings show that exposure to the different caregiving conditions produced a complex array of DNA methylation changes that vary between hippocampal region, sexes, treatment groups, and gene locus. For all conditions, it was found that there was no one CG site driving DNA methylation changes, with DNA methylation changes equally occurring across all CG sites within the examined regions of the *Bdnf* gene. Interestingly, more DNA methylation changes were observed in the VH. Specifically, maltreated females had significantly higher levels of methylated DNA at *Bdnf* exon I compared to cross-foster care and normal care controls, while maltreated males had significantly higher levels of methylated DNA at *Bdnf* exon IV compared to cross-foster care and normal care controls. Further, cross-foster care females had significantly higher levels of methylated DNA at *Bdnf* exon IV compared

to normal care controls. In the DH, the only significant finding was that normal-care males had significantly higher levels of methylated DNA at *Bdnf* exon IV compared to cross-foster care and maltreated rats. These differences could be attributed to the fact that there are differential functional effects of stress hormones in the DH and VH (Maggio & Segal, 2009, Hawley & Leasure, 2012).

These findings of differential epigenetic marking of the *Bdnf* gene are consistent with other reports, where environmental stimuli and conditions have been shown to produce a complex pattern of DNA methylation changes that vary across *Bdnf* gene loci (Blaze et al., in press, Roth et al., 2009; Lubin et al., 2008; Fuchikami et al., 2011) or within specific regions of the hippocampus for a given exon (Roth et al., 2011). In addition to a previous report of stress-induced changes at exons I and IV between sexes in the medial prefrontal cortex in the same model (Blaze et al., in press), my data now demonstrate that there are divergent trajectories with regards to exon I and IV methylation between sexes in the DH and VH. These observations are also consistent with previous research demonstrating sex-specific differences in basal expression of chromatin-regulating enzymes and other experience-induced changes in DNA methylation (Auger, Jessen & Edelmann, 2011; Nugent & McCarthy, 2011). The basis of our sex-specific patterns could reflect a number of factors, including differential stimulation of males and females by dams within our treatment conditions. It has been found that rat mothers typically groom the anogenital region of males more than females, so it is possible that variations in maternal care may be contributing to sex differences in behavior by epigenetically modifying the genome. This would suggest that maternal behavior might alter DNA that would later result in divergent male and female gene expression levels (McCarthy et al., 2009).

4.3 Gene Expression Patterns are Influenced by Early-Life Caregiving Experiences

As one way to explore the functional relevance of our hippocampal DNA methylation changes, basal changes in gene expression were also measured. In the VH, maltreated females had significantly lower levels of *Bdnf* gene expression in comparison to cross-foster care females. In the DH, maltreated females had lower levels of *Bdnf* gene expression compared to controls and maltreated males. It is likely that exon-specific transcripts are too altered, especially given the methylation results in regards to exon I and IV. This possibility was not examined here but could be a useful extension to the study in order to strengthen the causal relationship between DNA methylation and gene expression patterns.

4.4 *Bdnf* DNA Methylation and Gene Expression Patterns Could Map Onto Alterations in Hippocampal-Dependent Behavior

My project only focused on a PN90 cohort, but using a lifespan approach, the lab is currently assessing whether these DNA methylation and gene expression patterns developed early (in infancy) or emerged later in life. The lab is also exploring whether these patterns map onto changes in hippocampal-dependent behavior between our treatment groups and sexes. Several groups have investigated the consequences of early-life stress on hippocampal function. For example, rats subjected to recurrent

maternal separation have been shown to have severe spatial memory impairments in the Morris watermaze task at 12 months of age (Brunson et al., 2005). The same study also found deficits in an object recognition task, namely that the stressed rats failed to distinguish a novel object from one they had seen the previous day, further strengthening the claim that hippocampal cognitive function is impaired in middle-aged rats stressed early in life. These data suggest that rats exposed to our adverse caregiving conditions might too show deficits in learning and memory function.

4.5 Transgenerational Effects of Early-Life Caregiving Experiences

The data I've collected contribute to the emerging insight of the ability of early-life adversity to epigenetically modify the genome. Another future direction for this research would be to assess whether these hippocampal modifications are present in future generations. A previous study using the same maternal care paradigm showed that maltreated-induced alterations in *Bdnf* methylation were passed on the next generation of infants (Roth et al., 2009). Recognizing the biological consequences and transgenerational impact of abuse has critical importance for both disease research and public health policy. Additional work is needed to understand the mechanisms by which HPA axis stress-induced changes and the mental and physical consequences of these changes pass between generations, but it is undeniable that the effects of abuse are not limited to the immediate victims (Neigh, Gillespie & Nemeroff, 2009).

4.6 Reversibility of Effects of Early-Life Caregiver Experiences

Although the current study demonstrates epigenetic consequences of early-life experiences, this does not mean that developmental outcomes are insensitive to later-life environmental conditions. In the case of DNA methylation, there is evidence that pharmacological manipulation of the epigenome can reverse the effects of early-life caregiving experiences. One study previously mentioned from Michael Meaney and his colleagues indicated that adult offspring who had received low levels of maternal care in infancy that were given a 2-week treatment with trichostatin A, a drug that promotes histone acetylation and reduces methylation, had decreased hippocampal *GR* methylation, increased *GR* mRNA, and decreased responsivity to stress (Weaver et al., 2004). In contrast, adult offspring who had received high levels of maternal care in infancy that were given a 2-week treatment with methionine, which results in increased availability of methyl groups, had increased hippocampal *GR* methylation, decreased *GR* expression and a heightened behavioral response to stress (Weaver et al., 2005).

Further, Tania Roth has demonstrated a causal relationship between the observed epigenetic markings and deficits in gene expression in a previous study with the same model I used here (Roth et al., 2009). Adult male and female rats that had experienced maltreatment were exposed to a seven-day drug infusion treatment regimen of a DNA methylation inhibitor called zebularine, and this drug treatment regimen was able to rescue both the abnormal DNA methylation and gene expression patterns induced by adverse maternal care. Since past work in our lab and that of others has demonstrated that the epigenetic and behavior effects of early-life adversity are potentially modifiable, an important future direction would be to assess the

reversibility of the DNA methylation and gene expression patterns that I found in this study. Although the aforementioned studies have provided encouraging evidence, the data is incomplete and the ability to interpret the clinical relevance is challenging. This is because these pharmacological manipulations have not been assessed on a long-term scale, and the potential for these drugs to alter epigenetic mechanisms on a global scale has not been addressed either (Roth & Sweatt, 2010).

4.7 Considerations of the *Bdnf* Gene Polymorphism

A lot has been learned about epigenetics in mediating the long-term effects of early-life experiences, but most of the data has come from animal models in which the behavioral outcome is mostly constant and excludes confounds of genetic variability. In humans, it is important to be aware that genetic polymorphisms, or variations in specific DNA sequences within the population, exist and that they contribute to experiences producing a particular outcome in some people but not in others. They are suggested to add another level of complexity in understanding how behavioral outcomes are influenced by early-life experiences, and this can be exemplified by a number of studies (Roth & Sweatt, 2010).

With regards to the *Bdnf* gene, there is a common single-nucleotide polymorphism (SNP) that causes a valine (Val) to methionine (Met) substitution at codon 66. This SNP has been demonstrated to alter *Bdnf* mRNA, BDNF protein, and has been implicated in psychiatric disorders (Boulle, 2012). Specifically, one study found that *Bdnf* could influence the risk of depression by altering reactivity of the HPA axis (Schule et al., 2006). It was found that homozygous carriers of the Met/Met

genotype had significantly higher HPA axis activity during a dexamethasone challenge than patients carrying the Val/Val or Val/Met genotype. Additionally, another study found that the Val66Met polymorphism moderates the effects of early adverse experience on attention problems (Gunnar et al., 2012). Children carrying at least one copy of the Met allele were more sensitive to the time spent in an institution, and the longer the duration in institutional care, the higher number of attention problem symptoms. These studies and others reveal promising data, and it will be important in future studies to investigate the interactions of these genetic variables with experience-driven epigenetic changes, particularly by establishing animal models to facilitate this kind of study (Roth & Sweatt, 2010).

4.8 Other Genes and Epigenetic Mechanisms are Affected by Early-Life Caregiver Experiences

Although many studies have determined that the *Bdnf* gene is affected by early-life experiences, it is important to note that it is not the only gene altered in response to these experiences. One prime example is a recent genome-wide study of DNA promoter methylation in the hippocampus of individuals with a history of severe child abuse (Labonté et al., 2012). 362 differentially methylated promoters (248 hypermethylated and 114 hypomethylated) were identified in individuals with a history of abuse compared to controls (with no history of child abuse). Genes involved in cellular and neuronal plasticity were some of the most significantly differentially methylated, and overall their findings suggest that a number of genes are subject to environmental programming and could be involved in producing risk or resiliency to

adverse outcomes later in life. Future work with this model should look at the ability of our caregiver manipulations to epigenetically alter additional gene loci within the hippocampus.

It should also be noted that although there is ample evidence that early-life experiences produce lasting changes in DNA methylation, it is not the only epigenetic mechanism that could be altered by these experiences. There is significantly less research involving early environmental effects on histone modifications such as acetylation, methylation, and sumoylation, and it will be an important future direction to address these aspects of epigenetic modifications further. It is probable that both DNA methylation and histone modifications are involved in mediating the long-term effects of early environmental influences (Roth & Sweatt, 2010).

4.9 Conclusion

It is clear that behavior of the mother towards her offspring can produce epigenetic modifications and changes in expression of the *Bdnf* gene that extend beyond the period of maternal care. Further research with this model and in humans will help us understand a biological basis of the prolonged effects of early-life experiences on gene regulation and behavioral outcomes. Knowing how these experiences confer increased risk for mental disorders, how they are maintained and how they could be reversed, is gaining attention in psychiatry and developmental psychology, and should help establish new guidelines for better therapeutic interventions of these disorders.

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