ASSESSMENT OF EPIGENETIC CHANGES ASSOCIATED WITH CAREGIVING IN THE MEDIAL PREFRONTAL CORTEX OF ADOLESCENT RATS

by

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ABSTRACT

An early stressful environment, such as child abuse, has been shown to have profound effects on neurodevelopment and later behavior. The field of epigenetics has provided insight into how long-term changes in gene expression and behavior can be catalyzed by early-life stressful experience and maintained throughout one's lifetime by way of chromatin modifications. In the current study, we aimed to characterize epigenetic changes to *Bdnf* and *Reelin*, genes important in development and plasticity, in the adolescent medial prefrontal cortex (mPFC) after manipulation of infant-caregiver experiences. Using a within-litter design, rat pups were exposed to either an abusive or nurturing caregiver (maltreatment or cross-foster care conditions, respectively) for 30 minutes per day during the first postnatal week. Biochemistry results indicate a significant decrease in methylated DNA associated with *Bdnf* exon IV for maltreated females and an increase in methylated DNA associated with *Bdnf* exon I for maltreated males. Gene expression assays detected a decrease in the expression of *Reelin* and *Bdnf* in males who experienced nurturing care outside of the home cage (cross-foster care). The data demonstrate differential epigenetic effects of early life stress in the mPFC that are present past the initial period of manipulation and are specific to caregiving environment, sex, and gene locus.

Chapter 1

INTRODUCTION

Human Research on Child Abuse and Neglect

Worldwide, physical child abuse is estimated to affect 25-50% of all children as stated in a World Health Organization (2010) study, although it is difficult to measure such a complex statistic worldwide due to lack of sufficient data from many middle and lower income countries. In higher-income countries, child abuse and neglect (child maltreatment) is estimated to affect 4-16% of children (Gilbert et al., 2008). In the United States alone, child maltreatment affects approximately 12.3 children out of every 1000 (U.S. Department of Health and Human Services, 2002). Statistics of child maltreatment encompass physical abuse, sexual abuse, emotional abuse, and neglect of children by their parents or caregivers. It is important to note that the data above were determined by only the number of investigated child maltreatment cases. Unfortunately not all child maltreatment cases are investigated, and therefore it is believed that the numbers reported are much lower than the actual number of cases (Cicchetti & Toth, 2005).

Child maltreatment is a tragic and serious public health and social welfare problem estimated to directly and indirectly cost United States citizens between $56 and $94 billion annually. An Economic Impact Study by Wang and Holton (2007) estimates the overall cost to be even higher, at $103 billion each year. Direct costs include hospital bills incurred from physical abuse, police enforcement, and court action. Indirect costs are attributed to long-term costs of juvenile delinquencies,

special education, mental and physical health care, lost productivity to society, and therapy due to psychological disorders such as mood and anxiety disorders (Wang & Holten, 2007; Cicchetti & Toth, 2005). Although monetary costs are very steep, the psychological toll on the victims of child maltreatment is profound and can cause mental and emotional repercussions lasting long after the initial maltreatment. Children who have been emotionally, physically or sexually abused both early and later in childhood may display increased antisocial behavior according to self-reports or peer and teacher measures when compared with non-abused children of the same age and gender (Cicchetti, Rogosch & Thibodeau, 2012). A multitude of other reports indicate that child maltreatment is associated with a higher risk of developing anxiety, mood, and personality disorders soon after the abuse (Famularo, Kinscherff & Fenton, 1992; Pelcovitz et al. 1994), and these psychiatric disorders may persist throughout or not develop until much later in life (Saunders et al. 1992; Mullen et al., 1996; Stein et al., 1996; McCauley et al., 1997; Cicchetti & Toth, 2005). Of course not all victims of caregiver maltreatment develop psychopathology. Some children are remarkably resilient to the effects of child maltreatment. Resiliency can be defined as an unexpected but successful adaptation after experiencing an extremely adverse and stressful environment (Cicchetti & Blender, 2006). Three major suggested psychosocial factors of maltreated children who develop resiliency are the child's individual attributes, aspects of their families, and characteristics of their broader environments (Masten & Garmezy, 1991).

Research in the field of childhood adversity reflects observed cognitive and neurobiological impairments. Cognitive deficits associated with child maltreatment include impairments in academic performance and IQ (Majer et al., 2010; Navalta et

al., 2006), significant impairments in short and long-term memory (Beers & De Bellis, 2002; Bremner et al., 2004), working memory impairments (Raine et al., 2001), auditory and visual attention deficits (DePrince, Weinzierl, & Combs, 2009), decreased inhibitory control (Navalta et al., 2006), and poor emotion processing (Kim & Cicchetti, 2010; Pollak & Tolley-Schell, 2003). In many reports, the severity of the maltreatment has been related to the level of cognitive deficits. The disruption of cognitive development may reflect impaired neural function in maltreated children.

From a neurobiological perspective, differences between maltreated and non-maltreated children have been reported in neuroendocrine regulation and neuroimaging studies. All forms of child maltreatment can cause high levels of stress in the victims of abuse. The hypothalamic-pituitary-adrenal (HPA) axis is a neuroendocrine system which regulates stress through a negative feedback loop. Briefly, the paraventricular nucleus of the hypothalamus releases corticotropin-releasing hormone (CRH), stimulating the secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland, which enters the blood stream causing the adrenal cortex of the adrenal gland to produce glucocorticoids, such as cortisol (Sawchenko et al., 1993). Cortisol (or corticosterone in animals) is responsible for completing the negative feedback system, inhibiting the further secretion of CRH and ACTH. The ability to release cortisol during periods of acute stress is necessary for survival and allows for the appropriate management of stressful experiences behaviorally and physiologically (Watts, 1996).

Chronic stressors, such as child maltreatment, cause sustained heightened cortisol levels due to hyperactivation of the HPA axis, as observed in children who were physically and sexually abused (Cicchetti & Rogosch, 2001). Abused children

diagnosed with depression (Hart, Gunnar & Cicchetti, 1996) were observed to have higher salivary cortisol as were maltreated children diagnosed with PTSD (Carrion et al., 2002) in comparison to non-abused controls. Urine samples of maltreated children with PTSD showed elevated active, unbound (free) cortisol concentrations (De Bellis et al., 1994). Also, magnetic resonance imaging (MRI) showed greater increases in pituitary volume with age in prepubescent maltreated children with PTSD (Thomas & De Bellis, 2004).

Different neurotransmitter systems are also affected by chronic stressors, such as child maltreatment. Specifically, intense anxiety activates the locus coeruleus, an area in the brain stem that produces norepinephrine causing a cascade of biological changes responsible for the fight or flight reaction. Within the brain during stressful events, the locus coeruleus also activates the amygdala, a brain region responsible for regulating emotion and anxiety, which in turn stimulates the HPA axis. At the same time, the amygdala also stimulates dopaminergic inputs to the medial prefrontal cortex through complex mechanisms, resulting in enhanced attention and cognitive processing during periods of stress (Bertolucchi-D'Angio, Serrano & Scatton, 1990). However, chronic stress has been strongly associated with decreased attention, difficulty learning new material, psychotic symptoms, and paranoia in children, due to excess dopamine activity which in turn impairs prefrontal cortex functioning (De Bellis, 2005). To measure differences in brain volume of maltreated children, neuroimaging studies provide evidence that the mPFC is hyporesponsive whereas the amygdala is hyperresponsive in adults with PTSD and a history of child abuse (Bremner et al., 1999; Lanius et al., 2002; Rauch et al., 1996; Rauch et al., 2000; Shin

et al., 1999). Together, these studies demonstrate the strong association between early maltreatment and developmental changes in biological systems that regulate stress.

Non-Human Primates

Caregiver maltreatment is not unique to humans, and its presence in non-human primates has allowed further investigation of the neurobiological consequences of early-life adversity (Maestripieri & Carroll, 1998; Maestripieri, Wallen & Carroll, 1997). It is estimated from various field studies that a small proportion (5-10%) of a mothers within rhesus and pigtail macaque social groups physically abuse their infants by hitting, biting, dragging, sitting, or crushing them (Maestripieri, 1999; Sanchez, Ladd, & Plotskey, 2001). These abusive behaviors cause increases in infant stress, and in rare cases cause infant death. When parenting styles were more closely examined, abusive rhesus macaque mothers were more rejecting in their parenting style spending less time in contact with infants and were less protective of infants, compared with non-abusive rhesus macaque mothers that were nurturing in parenting style, and protective in behavior (Maestripieri, 1998). Infants who experienced caregiver abuse early in life exhibited delayed social development as shown by delayed independence from their mothers and decreased play and exploration. Abused infants were also observed to be more distressed and irritable as demonstrated by higher rates of distress calls, screams, and tantrums, compared with non-abused controls (Maestripieri et al., 2000; McCormack, Sanchez, Bardi & Maestripieri, 2006).

Early life caregiver maltreatment of non-human primates is also associated with neurobiological and neuroanatomical differences. In response to CRH challenges measured at 6-month time intervals during the first three years of life, abused infant rhesus macaques were observed to have greater cortisol responses to CRH than non-

abused controls (Koch, McCormack, Sanchez, & Maestripieri, 2012). These results demonstrate that long-term alterations in neuroendocrine function may be one way that infant abuse results in psychopathologies. Other long-term stressors such as maternal deprivation, have shown associated deficits in attention and motivation representing aberrant prefrontal cortex function (Beauchamp & Gluck, 1988; Beauchamp, Gluck, & Lewis, 1991), and observed decreases in white prefrontal cortex matter, as measured by structural MRI (Sanchez, Hearn, Do, Rilling & Herndon, 1998).

Rodent Models of Early Life Adversity

Although human and non-human primate research is invaluable to the field of child abuse and neglect, rodent models allow the utilization of intrusive techniques to directly study how brain development and subsequent behavior are influenced by early-life stress. Rodent studies have been able to replicate many of the findings from human and non-human primate studies, giving support for the validity of rodent models. There are several rodent models reported in the literature, but of particular relevance to my thesis work is utilizing resource deprivation (nesting material of nursing mothers). Resource deprivation is an experimental design which elicits unpredictable and aversive maternal behavior from rodent mothers toward their infants (Ivy, Brunson, Sandman, & Baram, 2008; Rice, Sandman, Lenjavi, & Baram, 2008; Roth & Sullivan, 2005; Roth et al., 2009). The more limited the amount of shavings allotted, the more the interactions between mother and infant rodent are disrupted. Resource deprived mothers display higher rates of fragmented care of pups, as measured by the number of maternal exits from nesting area, compared with non-resourced deprived controls (Ivy et al., 2008). Manipulation of this variable, in

conjunction with environmental novelty, also evokes caregiver maltreatment (Roth & Sullivan, 2005; Roth et al., 2009). Long-term changes in behavior and physiology have also been observed in pups that experienced fragmented maternal care. For example, the Morris water maze and the object recognition test, measures of hippocampal-dependent learning and memory function in rodents, showed that adult mice subjected to an early stressful environment, specifically abnormal and fragmented maternal interactions, had deficits in learning and memory compared with controls. Additionally, mice who experienced fragmented maternal care within the homecage had significantly higher levels of blood plasma corticosterone levels as adults, decreased CRH mRNA levels of the hypothalamic paraventricular nucleus, and lower body weight, indicating that the pups that experienced fragmented maternal care due to resource deprivation displayed neurobiological abnormalities and behavioral deficits in adulthood (Rice et al., 2008; Ivy et al., 2008).

Epigenetics

The term 'epigenetics', literally meaning "above the genome", was coined by Conrad Waddington, known for pioneering the integration of embryology, evolution, and genetics. Epigenetics broadly encompasses events that can not be explained by Mendelian genetic principles. Advances in embryology led researchers to believe that epigenetic regulation only occurred during cellular differentiation and development (Holliday, 2006). While epigenetic modifications are essential for the specification of cell types (cellular differentiation) during early development (Morgan, Santos, Green, Dean & Reik, 2005), evidence of epigenetic regulation present in mature cells has been uncovered in more recent research and has helped revolutionize the fields of neuroscience, psychology, and psychiatry.

Today, the epigenetics research studies how gene expression can be further regulated by mechanisms other than changes in nucleotide sequence, mechanisms such as DNA methylation and histone acetylation. Epigenetic mechanisms provide an explanation for why monozygous (identical) twins, who share the exact same genotypes, are discordant in later phenotypic outcomes. For example, in 2005, Fraga and colleagues demonstrated that as human monozygous twins aged and thus experienced increasingly different environments, their divergent outcomes were attributed to differences in epigenetic patterning (Fraga et al., 2005). Epigenetic regulation has also exemplified one way in which genes and a stressful environment early in life interact to produce different phenotypes. In the study of an early adverse environment associated with persistent epigenetic modifications in humans, researchers found that people who were prenatally exposed to the Dutch famine (1944-1945), had significantly less methylation of the imprinted insulin-like growth factor II (IGF2) gene compared with their unexposed same-sex siblings (Heijmans et al., 2008). These modifications persisted even 60 years after the famine occurred, providing a mechanism of how the early environment can moderate genes and cause long lasting biological changes.

Mechanisms of DNA Methylation

Scientists are currently investigating the complex molecular mechanisms activated during DNA methylation. The process of DNA methylation involves the addition of methyl groups (CH_3) to the cytosine of cytosine-phosphate-guanine (CpG) dinucleotide sites, which was originally proposed by two independent research groups (Holliday & Pugh, 1975; Riggs, 1975).

Areas of the genome containing many CpG sites are referred to as CpG islands, and the DNA methylation of these CpG islands is usually correlated with transcriptional repression (Miranda & Jones, 2007). Enzymes known as DNA methyltransferases (DNMTs), such as DNMT1 and DNMT3a, catalyze the process of DNA methylation (Goll & Bestor, 2005; Moore, Le, & Fan, 2013). DNMT1 is a maintenance methyltransferase that adds methyl groups to the complimentary DNA strand of identified hemimethylated DNA, thereby maintaining previously created methylation patterns. On the other hand, DNMT3a is a *de novo* methyltransferase that establishes new patterns of DNA methylation by adding methyl groups to previously unmethylated DNA. The active demethylation (i.e. the removal of methyl groups) of cytosines is thought to occur via a DNA repair mechanism catalyzed by the protein Gadd45b (growth arrest DNA-damage-inducible beta) (Ma, Guo, Ming & Song, 2009). The demethylating actions of Gadd45b have been observed at genes critical for adult neurogenesis such as the *Brain-derived neurotrophic factor* (*Bdnf*) gene and the *Fibroblast-growth factor* (*FGF*) gene (Ma et al., 2009).

The majority of current literature views DNA methylation as an epigenetic mechanism responsible for gene suppression. Gene silencing may occur through the interference of transcription factors by methyl groups, and may also be mediated by the binding of methyl-CpG-binding-protein 2 (MeCP2), which can recruit histone deacetylases (HDACs) and other co-repressors to inhibit gene transcription. There is evidence however that MeCP2 can recruit transcription factors such as CREB1 and other co-activators to promote gene transcription and activation (Chahrour et al., 2008). The repressive view of DNA methylation thus may be more of generalization than .

Early Life Experience and Epigenetic Modification

Rodent studies have suggested a causal relationship between the quality of the caregiving environment and the DNA methylation patterns within the brain. In a revolutionary paper from Michael Meaney's laboratory, naturally occurring variations in maternal caregiving, specifically levels of arched back nursing (high/low ABN) and licking/grooming of pups (high/low LG), demonstrated that infant experience with low levels of nurturing care (low LG-ABN) resulted in offspring that had significantly more DNA methylation of the *glucocorticoid receptor* (*GR*) gene promoter in the hippocampus, compared with infants exposed to nurturing care (high LG-ABN) (Weaver et al., 2004). They also demonstrated that these differences in DNA methylation persisted into adulthood. The *GR* gene expression in the hippocampus was also correlated with the experienced maternal care condition, where high LG-ABN offspring had increased levels of *GR* gene expression, compared with low LG-ABN offspring who had significantly decreased *GR* gene expression. As adults, the offspring of low LG-ABN mothers had higher anxiety responses, compared with moderate levels of anxiety of high LG-ABN offspring, as measured by corticosterone levels from restraint stress. The methylation and gene expression effects of maternal caregiving type were also shown to be independent of birth mother level of LG as shown via cross-fostering experiments to be independent of the birth mother (switching to the opposite caregiving type mother 12 hours after birth).

Findings from this rodent model have also been translated to humans. Specifically, one study showed that suicide completers with a history of childhood abuse had higher DNA methylation of the *GR* gene promoter (*NR3C1*, the human equivalent of the rat exon I_7 in the glucocorticoid receptor promoter, investigated in the study by Weaver et al., 2004) in hippocampal brain tissue, compared with suicide

completers that were not abused and non-suicide controls (McGowan, et al., 2009). Researchers also found significantly decreased expression of the hippocampal glucocorticoid receptor in abused suicide victims compared with non-abused suicide victims and controls. Together, the rodent model and human results suggest a common effect of caregiving on the epigenetic regulation of hippocampal glucocorticoid receptor expression.

Later work that utilized the same Meaney laboratory rodent model (Bagot et al., 2012; Champagne et al., 2006; Weaver et al., 2006; Zhang et al., 2010), or infant-maternal separation paradigms (Franklin et al., 2010; Murgatroyd et al., 2009) have extended the link between caregiver experiences and epigenetic programming to other gene loci within the hippocampus and in other brain regions, such as the HPA axis nuclei. For example, rodents who experienced low levels of nurturing care (low LG) early in life were observed to have increased DNA methylation of the *glutamic acid decarboxylase 1* (*GAD1*) promoter in hippocampal tissue, which codes for a rate-limiting enzyme in GABA neurotransmitter synthesis, compared with high LG offspring who had decreased *GAD1* promoter DNA methylation (Zhang et al., 2010). Additionally, high LG offspring showed increased *GAD1* mRNA expression while low LG offspring showed decreased *GAD1* mRNA expression. Also in the hippocampus, variations in maternal care were associated with epigenetic regulation of a different gene, *Grm1* which encodes for metabotropic glutamate receptor 1 (mGluR1) (Bagot et al., 2012). Researchers found that high LG offspring had decreased DNA methylation of the *Grm1* gene, along with an increase in the mGluR1 mRNA expressed compared with offspring of low LG mothers who showed increased DNA methylation of *Grm1* with decreased mGluR1 mRNA levels.

Maternal care has also been associated with epigenetic changes in the *estrogen receptor-α1b* (*ERα1b*) promoter in the medial preoptic area (MPOA) of female offspring (Champagne et al., 2006). Differentiated gene expression of the *ERα* mRNA levels was observed, specifically infant female offspring of high LG mothers showed increased *ERα* expression, compared with low LG female offspring who had decreased *ERα* expression. DNA methylation of the *ERα1b* promoter region demonstrated effects correlated with *ERα* mRNA levels, such that female adult rats experiencing high LG as infants had decreased DNA methylation of the *ERα1b* promoter while female low LG offspring had increased levels of *ERα1b* promoter methylation. The data also demonstrated epigenetic effects that are sex-specific and persist past the initial period of maternal caregiving. These results also demonstrate the inverse relationship that DNA methylation and gene expression have, such that increases in DNA methylation are associated with decreases in gene expression and decreases in DNA methylation are associated with increases in gene expression.

Another type of early life adversity rodent model, maternal deprivation, has also shown associated behavioral and epigenetic effects (Franklin et al., 2010; Mugatroyd et al., 2009). During the first two postnatal weeks, mice were exposed to unpredictable maternal separation combined with unpredictable maternal stress (MSUS) or left undisturbed with their biological mother, serving as the control group (Franklin et al., 2010). In adulthood, MSUS offspring showed increased depressive-like behavior compared with controls, and these behavioral effects were expressed by the first and second generation offspring of males subjected to MSUS, even though the offspring were raised normally. MSUS was also found to alter the profile of DNA methylation in the promoter region of several candidate genes in the germline of

affected males, and similar changes in DNA methylation was found in the brains of first generation females of male MSUS offspring. Effects were observed to be sex-dependent and transgenerational, possibly attributed to the differential DNA methylation of candidate genes. Maternal separation was also observed to invoke hypersecretion of corticosterone and deficits in passive stress coping and memory in the infants exposed to the early life stress paradigm (Murgatroyd et al., 2009). These changes in stress response were also associated with epigenetic changes in the paraventricular nucleus (PVN) of the hypothalamus. Infants who experienced unpredictable maternal caregiving showed increased expression of the *arginine vasopressin* (*AVP*) gene, and decreased DNA methylation regulatory sites in the *AVP* gene. These two studies illustrate the fact that other forms of early-life adversity, such as unpredictable maternal care, can influence the epigenetic regulation of gene loci in brain areas relevant to stress response.

Studies have shown a relationship between epigenetic regulation of genes within the prefrontal cortex in the context of memory in adult animals (Bredy et al., 2007; Miller et al., 2010; Sui, Wang, Ju, & Chen, 2012), but the relationship between the quality of maternal care and epigenetic regulation of genes within the PFC has only recently started to gain attention (Provencal et al., 2012; Roth et al., 2009). The PFC is a brain region that is quite sensitive to the harmful effects of early life adversity (De Bellis et al., 2002; Hanson et al., 2010). For instance in children with maltreatment-related PTSD, a MRI study showed they had smaller PFC and PFC white matter volume compared with control subjects that were sociodemographically matched, illustrating that maltreatment is associated with aberrant PFC development (De Bellis et al., 2002).

Focused on the same brain area but in a rodent model, prior work in Tania Roth's lab has demonstrated that caregiver maltreatment of infant rat pups during the first postnatal week caused increased methylation of the *Bdnf* gene in the whole PFC, and a concomitant decrease in *Bdnf* mRNA expression of adult rats (Roth et al., 2009). The *Bdnf* gene encodes a protein that is essential for neurogenesis, neuron proliferation, differentiation, and survival (Barde, 1994; Connor & Dragunow, 1998; Zheng, Zhou, Moon, & Wang, 2012), and has been linked to various psychiatric disorders (Boulle et al., 2012; Calabrese, Molteni, Racagni & Riva, 2009; Autry & Monteggia, 2012). Male and female maltreated rats had increased levels of *Bdnf* exon IX methylation that persisted through infancy, adolescence and adulthood. Methylation changes at exon IV of the *Bdnf* gene did not emerge until adulthood. The effects of maternal caregiving on *reelin* gene expression and DNA methylation were also studied. The *reelin* gene encodes for reelin, an extracellular matrix protein important for neuronal migration during early neuronal development long-lasting structural changes in neurons (Pesold et al., 1999). *Reelin* is a gene likewise implicated in psychiatric disorders (Abdolmaleky et al., 2005; Grayson & Guidotti, 2012; Tissir & Goffinet, 2003), and its methylation patterns can be influenced by the environment (Sui, Wang, Ju, & Chen, 2012; Martinez et al., 2011). There were no significant differences found in adult PFC *reelin* mRNA levels between maltreated or rats with a normal upbringing, nor were there changes in *reelin* DNA methylation detected.

The PFC can be divided into many distinctive functional parts, and several lines of work show a correlation between early-life adversity and the malfunctioning of the medial prefrontal cortex (mPFC) in humans (Bluhm et al., 2009; Philip et al.,

2013). The mPFC is known to be involved in regulating the HPA axis activity under basal and stressful conditions in rodents (Diorio, Viau & Meaney, 1993). For example, lesions of the mPFC were associated with significantly increased plasma levels of both ACTH and CORT following acute (20 minute) restraint stress. Also, crystalline CORT implants into the mPFC significantly reduced plasma ACTH and CORT responses to restraint stress. These findings suggest that the mPFC is a target for the negative-feedback effects of glucocorticoids on stress-induced HPA activity.

Research Aims

To our knowledge, there have been no studies that have investigated the link between early adverse care and epigenetic changes in the medial prefrontal cortex. It is possible that early-life maltreatment could contribute to long-term effects on the structure and function of the mPFC via epigenetic mechanisms, as the precise biological basis has yet to be discovered. The overall goal of my thesis research thus was to examine whether exposure to adverse caregiving could alter DNA methylation patterns within the mPFC. The research aims specifically were: 1) to further characterize maternal behavior within the lab's caregiving model; 2) to characterize epigenetic changes at *Bdnf* gene loci within the rat adolescent mPFC; and 3) to characterize differences in gene expression within the rat adolescent mPFC.

Chapter 2

METHODS

Subjects

Outbred Long-Evans rats from Harlan Laboratories were housed in polypropylene cages (18”x9”x8”) with plenty of wood shavings 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. The dams all had previously given birth to at least one litter of pups before the experiment, to ensure that no first-time mothers were part of the study. Postnatal day (PN) 0 represents the day the pups were born. 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 experiment.

Experimental Design

Using a within-litter design, rat pups were consistently exposed to either an abusive caregiver (maltreatment condition), nurturing caregiver outside the homecage (cross-foster care, CFC), or nurturing care within the homecage (normal maternal care, NMC) for 30 minutes per day during the first postnatal week (PN1-PN7). In the maternal maltreatment condition, non-biological lactating mothers were given only five minutes to habituate to a novel environment and were allotted only a very small amount (100 mL) of nesting material. This novel environment consisted of a different

black plastic container (18”x12”x18”) and was located in a room separate from the rat colony. Lactating dams received up to four pups (two males and two females) to care for during a 30 minute session. In the CFC condition, non-biological lactating dams were given one hour to habituate to a new environment and were given plenty of shavings for nesting prior to receiving up to four pups (two males and two females). The new environment consisted of a black plastic container (18”x12”x18”) with at least a 2 cm layer of wood shavings on the chamber floor, and was located in a room separate from the rat colony. In the NMC condition, which served as a control, up to four infant rats (two males and two females) which were only weighed and marked for identification, remained in their usual home cage environment with their biological mother. All experimental chambers were maintained between 24-29 ^{o}C, as measured by a digital thermometer. After the thirty minute exposure session, experimental pups were removed from the test chamber and reunited with their biological mother. Maltreatment and cross-foster dams were also returned with their biological pups after each exposure session. Besides weekly cage changes, the pups were undisturbed until PN21 when they were weaned and housed in same-sex pairs until adolescence (PN30) when brains were removed.

Maternal Behavior and Pup Response

Each day during the manipulation, audible and ultrasonic vocalizations (at a frequency of 40 kHz) were recorded using a SONY audio recording device and a bat detector (Batbox III D, NHBS Ltd., UK), while maternal 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 coded the vocalizations and behavior. Each recording was coded twice by different research

assistants to ensure inter-rater reliability. The audible and ultrasonic pup vocalizations were marked during each one minute interval if a distinctive pup vocalization was heard, then averaged across the week of thirty minute exposures. Maternal and infant behavior were observed during five minute intervals of the thirty minute exposure session, in which coders indicated if nurturing behaviors (such as pup licking, nursing, or grooming) or aversive behaviors (such as pup dragging, stepping on, or rough handling) were observed in each five minute time bend. Behaviors were marked using a list of clearly defined nurturing and aversive maternal behaviors, and the observations were averaged across the seven exposure days.

Biochemical Assays

On PN30 the adolescent rats were anesthetised using isofluorane and brains were removed and sliced using a 1 mm brain matrix. Slices were subsequently flash frozen on untreated slides with 2-methylbutane, and placed in a -80^{o}C freezer until later processing. The mPFC (bilateral prelimbic and infralimbic tissue) was dissected on dry ice using stereotaxic coordinates. After mPFC removal, both DNA and RNA were extracted using an AllPrep DNA/RNA kit (Qiagen Inc., Valencia, CA). Purified DNA and RNA were analyzed for quantification by measuring concentration and quality of nucleic acid with a spectrophotometer (NanoDrop 2000). To assess DNA methylation, purified DNA from mPFC tissue underwent bisulfite treatment using an EpiTect bisulfite kit (Qiagen Inc., Valencia, CA), in which unmethylated cytosines were converted to uracil. After that methyl-specific primers targeted methylated and unmethylated CG dinucleotides at regulatory exons I and IV within the *Bdnf* gene to assess methylation status through methylation specific real-time PCR (MSP) (Bio-Rad CFX96).

For gene expression assays, extracted RNA from mPFC tissue was reverse transcribed into cDNA using a reverse transcription kit (Qiagen Inc., Valencia, CA). During real-time PCR (Bio-Rad CFX96) reactions, cDNA was amplified with Taqman probes (Applied Biosystems) to target the mRNA of *Bdnf* (exon IX), *Reelin* and *Tubulin* (as a reference) genes. All target genes were compared with *Tubulin* as a reference gene. The *Tubulin* gene codes for a protein necessary for cell structure and support, and it is widely expressed in almost all cells, making it a viable reference gene. Every reaction for MSP and gene expression was run in triplicate. Agarose gel electrophoresis and melting curve analysis (MSP only) were performed to confirm product specificity (data not shown).

Statistical Analysis

One or two-way ANOVAs with post hocs, two-tailed unpaired t-tests, and one-sample t-tests were used to analyze data. Significance was set at $p \leq 0.05$, although non-significant trending data with $p < 0.1$ are reported here as well. Fold changes of the experimental groups (maltreatment or cross-foster care) with respect to the control group (normal maternal care) were calculated using the comparative Ct method for both MSP and gene expression experiments. For the MSP data, a methylation index (MI) was calculated by dividing the fold change value for the methylated primer set by the fold change value for the unmethylated primer set as discussed in a prior study (Sui et al., 2012). It should be noted that my project is part of a larger research effort in the lab to characterize epigenetic changes in PN8, PN30, and PN90 animals exposed to the different caregiver conditions. The maternal behavior and infant vocalization data thus reported here are from a larger cohort of animals used to generate animals for all three age groups and are not exclusive to the ten adolescent

litters that I used for my gene expression and DNA methylation analyses. The computer program PRISM was used to help perform statistical tests and aid in the creation of graphs.

Chapter 3

RESULTS

Caregiving Behaviors

To manipulate early-life caregiving experiences, infant male and female rats were repeatedly exposed to maltreatment outside the homecage, or nurturing care either outside the homecage (CFC) or inside the homecage (NMC). ANOVA tests revealed a main effect of caregiving behavior ($F_{1,98}$=245.8, $p<0.001$) and a behavior by infant condition interaction ($F_{2,98}$=173.7, $p<0.001$) (Figure 1a). Within both the NMC and CFC groups, infants experienced high nurturing care levels with low levels of adversity, and the two conditions did not differ significantly ($p>0.05$). Infants who were maltreated experienced high levels of adversity with low levels of nurturing care ($p<0.001$). The most common maternal behaviors observed in both the NMC and CFC conditions were infant licking, grooming, crouching over the pups and nursing, and have the highest percentages of coded behavior (figures 1b and 1c). In the maltreatment condition (figure 1d), a higher incidence of aversive behaviors such as stepping on pups, dropping pups during transport, dragging pups while attached to the nipple, actively avoiding pups, and rough handling pups were observed along with a lower percentage occurrence of nurturing behaviors.

Infant Responses to Caregiving Conditions

Audible and ultrasonic (40 kHz) vocalizations of the infant male and female rats were recorded during each exposure session across the three caregiver conditions

(figure 2). Prior research has identified 40 kHz ultrasonic vocalizations as distress calls emitted by rat pups (Portfors, 2007). Using one-way ANOVAs, significant differences across treatment groups were found for both audible ($F_{2,40}$=7.29, $p<0.01$) and ultrasonic ($F_{2,20}$=110.5, $p<0.0001$) vocalizations. While there was no significant difference between NMC and CFC conditions in the audible and ultrasonic vocalizations emitted ($p>0.05$), infants in the maltreatment condition emitted significantly more audible and ultrasonic vocalizations compared with the NMC ($p<0.05$ audible, $p<0.001$ ultrasonic), and CFC conditions ($p<0.01$ audible, $p<0.001$ ultrasonic).

Bdnf DNA Methylation in Adolescence

After the caregiving manipulations, Methylation specific real-time PCR was used to examine the presence of methylated vs. unmethylated DNA associated with *Bdnf* exons I and IV in adolescent (PN30) rats (figure 3). One-sample and unpaired t-tests revealed significant differences in the DNA methylation levels for our maltreated-subjects. Specifically, there was increased methylation of DNA associated with *Bdnf* exon I in maltreated males when compared to NMC males (t_9=3.29, $p<0.01$). At *Bdnf* exon IV, however, there was less methylation in maltreated females in comparison to NMC females (t_9=6.65, $p<0.001$). A two-way ANOVA for each *Bdnf* exon indicated no significant main effect of infant condition, nor an infant by sex interaction (all p-values > 0.1). There was however, a borderline significant effect of sex for exon I ($F_{1,35}$=3.37, $p=0.07$).

Gene Expression in Adolescence

To determine if *Bdnf* DNA methylation changes also coincided with basal alterations in *Bdnf* gene expression, real-time PCR was again utilized (figure 4). One sample t-tests revealed no significant differences in *Bdnf* mRNA levels between maltreatment and NMC controls (all p-values > 0.1), but there was a significant decrease in *Bdnf* mRNA in CFC males in comparison to NMC males (t_9=3.9, p<0.01). A two-way ANOVA revealed no main effect of infant condition, sex, nor an interaction between the variables (all p-values>0.1).

To determine if our treatments might have affected basal levels of another plasticity related gene, *reelin* gene expression was also characterized in this adolescent cohort (figure 5). One sample t-tests revealed no significant differences in *reelin* mRNA levels between maltreatment and NMC controls (all p-values > 0.1). An unpaired t-test however, revealed a trending decrease in *reelin* mRNA in CFC males versus NMC males (t_8= t=2.19, p=0.06). A two-way ANOVA showed no main effect of infant condition, sex, or infant condition by sex interaction on *reelin* mRNA levels (all p-values > 0.1).

Chapter 4

DISCUSSION

Here I provide the first assessment of maternal caregiving behaviors on epigenetic alterations within the adolescent mPFC. I used a unique paradigm that exposed both male and female rats from the same litter to different but re-occurring caregiving conditions. Exposing infants outside of the homecage to adversity/adverse caregiving allowed for controlled exposure to maltreatment without the confounding variable of maternal milk deprivation (which might occur if the biological mother had been subjected to resource deprivation within the homecage). The inclusion of the CFC group allowed me to discriminate effects produced by exposure to maltreatment from those produced by exposure to another caregiver in a different environment or removal from the biological mother and the homecage.

Maternal behavior observations indicated that both dams within the NMC and CFC conditions exhibited high levels of nurturing care, while dams in the maltreatment condition displayed high levels of aversive care and low levels of nurturing care. Measurement of infant responses to maternal care showed that maltreated rat pups emitted significantly more audible and ultrasonic (40 kHz) vocalizations than infants in the NMC or CFC conditions. The aversive maternal behaviors towards rat pups observed in the maltreatment condition were elicited by limiting the lactating dam's nesting material while she was in a novel environment. The maternal behavior data in this study replicated previous findings from the lab

(Roth et al., 2009; Roth et al., 2005), and support those from others (Ivy et al., 2008; Raineki et al., 2010).

Data here also help extend the characterization of this model, by reporting behavior within the NMC condition (which was not previously reported) and measuring infant vocalizations across the three conditions. In both control conditions, the lactating females in the familiar environment with plenty of nesting material (CFC) and the lactating biological caregivers within their homecage (NMC), displayed similar levels and types of nurturing care. Additionally, rat pup vocalizations demonstrate that the infants responded differently to the three maternal care conditions, with infants in the maltreatment condition emitting significantly higher amounts of audible and ultrasonic vocalizations compared with infants within the NMC and CFC conditions. Maternal separation or extreme physical stress induces ultrasonic vocalizations from rat pups, and the purpose of the vocalizations is to communicate distress signals to the mother to elicit maternal action. Cold temperature is one physical stressor that has been shown to induce 40 kHz vocalizations in rat pups (Portfors, 2007). The significant vocalizations emitted by maltreated pups in this study do not likely reflect low body temperatures, as we found no difference in body temperatures between infants exposed to our maltreatment or CFC conditions (data not shown).

The main goal of this research project was to examine whether maternal caregiving during infancy could alter DNA methylation within the mPFC. Biochemical assays for two genes important for brain development and plasticity, *bdnf* and *reelin*, showed effects specific to gene locus, sex, and maternal caregiving condition. For the *bdnf* gene, adolescent females that experienced caregiver

maltreatment were observed to have decreased methylation at exon IV, whereas adolescent males experiencing caregiver maltreatment were observed to have increased methylation at *bdnf* exon I. These epigenetic patterns show that differences in *bdnf* DNA methylation are age, sex and exon specific. These findings of differential epigenetic marking of the *bdnf* gene are consistent with other reports, where environmental stimuli and conditions evoke a complex pattern of DNA methylation changes that vary across the numerous *bdnf* gene exons, particularly of exons I and IV (Fuchikami et al., 2011; Lubin et al, 2008; Roth et al., 2009). The stress-induced *bdnf* DNA methylation results at exons I and IV in the mPFC expand knowledge from a previous report by Roth and colleagues (2009) who found stress-induced changes at *bdnf* exon IX in the adolescent whole prefrontal cortex (but not exon IV), and furthermore show divergent methylation effects between sexes.

As mentioned previously, this project is part of a larger research effort in the lab to characterize epigenetic changes in PN8, PN30, and PN90 animals exposed to these caregiver conditions. Work currently in press (Blaze et al., 2013) showed that the methylation changes reported here were not present in infants (PN8), and thus emerged sometime between infancy and adolescence. The later emergence of epigenetic patterning has also been observed in the *arginine vasopressin* (*AVP*) gene in the paraventricular nucleus (PVN) of male mice subjected to periods of separation from the mother, which was attributed to reduced MeCP2 binding (a methyl-binding domain protein that binds to methylated DNA) that was already present early in development and thought to confer later methylation changes (Murgatroyd et al., 2009). The sex difference in methylation here may be consistent with other reports showing sex-specific differences in the baseline levels of chromatin-regulating

enzymes (Jessen & Auger, 2011; Nugent & McCarthy, 2011). Other factors could have influenced the observed sex-specific methylation patterns, such as differing amounts of maternal attention to males and females within treatment groups. For example, mothers spend more time in active nursing and anogenital licking male pups compared to female pups (Moore & Chadwick-Dias, 1986; Moore & Morelli, 1979; Richmond & Sachs, 1984). It is possible that dams licked and groomed males more than females, but in this study maternal behavior was coded for all pups in the condition and not for individual rat pups. Future studies should aim to measure male- versus female-directed behavior to determine if there are differences in caregiving behaviors toward sexes across the three conditions.

As one way to explore the functional relevance of the mPFC DNA methylation levels, basal differences in group gene expression were assessed using quantitative RT-PCR. No significant changes in total levels of *bdnf* mRNA (exon IX, which measures all *bdnf* transcripts without preference for particular exon-containing transcripts) were detected in maltreated-subjects at rest. While baseline differences were not detected for total *bdnf* transcript levels, it is possible that DNA methylation alterations could have affected levels of specific *bdnf* transcripts (such as exon I- or exon IV-containing mRNA transcripts). In addition, though no changes in methylation of DNA associated with exons I or IV were detected in CFC animals, CFC males were found to have less *bdnf*-IX containing transcripts at baseline. These results suggest that their changes in *bdnf* expression might be driven by methylation of other *bdnf* exons that were not examined (exon VI for example).

Although the methylation levels of DNA associated with the *reelin* gene were not assessed in this project, levels of *reelin* gene expression in the animals at rest were

examined. No significant differences in basal *reelin* gene expression were detected. Exposure to high levels of prenatal stress or maternal deprivation has been shown to alter hippocampal *reelin* gene expression (Qin et al., 2011; Gross et al., 2012), but the effects in the mPFC were not examined in these studies, and the effects of early-life stress on cortical *reelin* in general have received little attention to date (Matrisciano et al., 2012). These studies also focused on adult animals, and it is possible that though no effects for gene expression were detected in adolescent animals, examining gene expression in older animals would reveal treatment effects on gene expression. Not only are DNA methylation changes relevant to basal mRNA levels, changes would also likely be important for activity-evoked gene regulation. For instance, the ability of an adult animal to learn and form memories has been associated with activity-evoked demethylation of *reelin* within the PFC (Miller et al., 2010), and demethylation and methylation of *bdnf* loci within the hippocampus (Lubin, Roth & Sweatt, 2008; Mizuno, Dempster, Mill & Giese, 2012). In the mPFC, Sui and colleauges (2012) demonstrated that both *bdnf* and *reelin* undergo demethylation in response to long-term potentiation induction. If the epigenetic regulation of genes has an active part in modulating an animal's ability to respond to its environment and later experiences in life, then altered methylation patterns of genes induced by experiences early in development have the potential to later affect behavior. Although in this study activity-evoked gene changes were not investigated, it could too be a future avenue of research.

In conclusion, both clinical research and studies utilizing animal models illustrate a strong relationship between early-life stress and later behavioral abnormalities, such as impairments in cognitive function, stress vulnerability, and an

increase in depressive- and anxiety-like behaviors. While studies are just beginning to show the role of mPFC dysfunction in aspects of these behaviors, the underlying molecular mechanisms have not been characterized. This study contributes to the literature concerning epigenetic alterations associated with the effects of early-life adversity, and specifically provide empirical data showing the ability of caregiver maltreatment to produce aberrant methylation of DNA associated with the *bdnf* gene in the mPFC. Future directions of related research could investigate whether the DNA methylation changes relate to specific phenotypes. This could be explored by performing anxiety, mood, and memory tests on adolescent rats who were exposed to our caregiving paradigm as infants. In the larger picture, research focusing on the short- and long-term relationship between DNA methylation and behavioral trajectories may provide professionals with innovative ideas for the treatment and intervention with children who have experienced child abuse and neglect.

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Appendix

FIGURES OF RESULTS

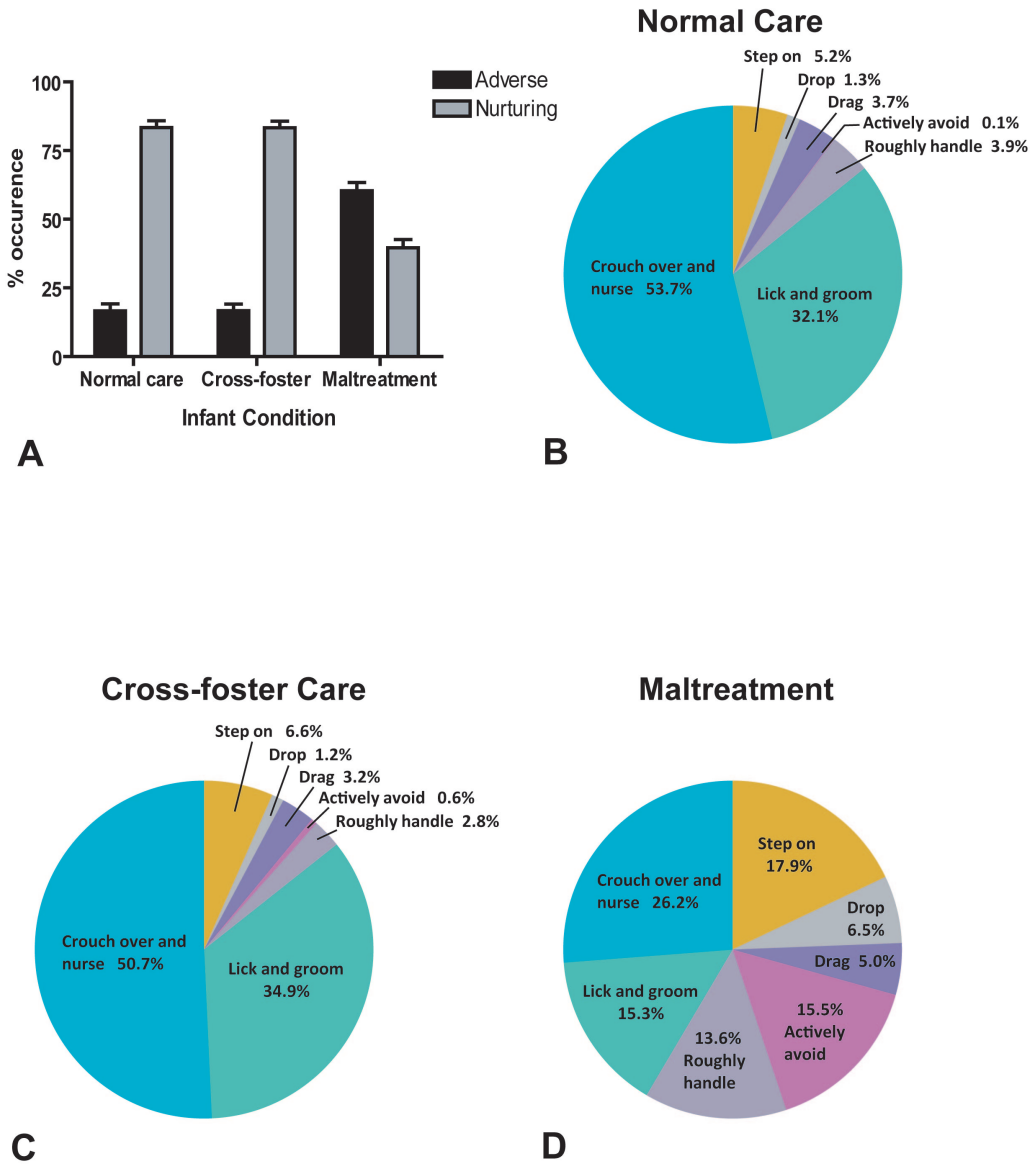
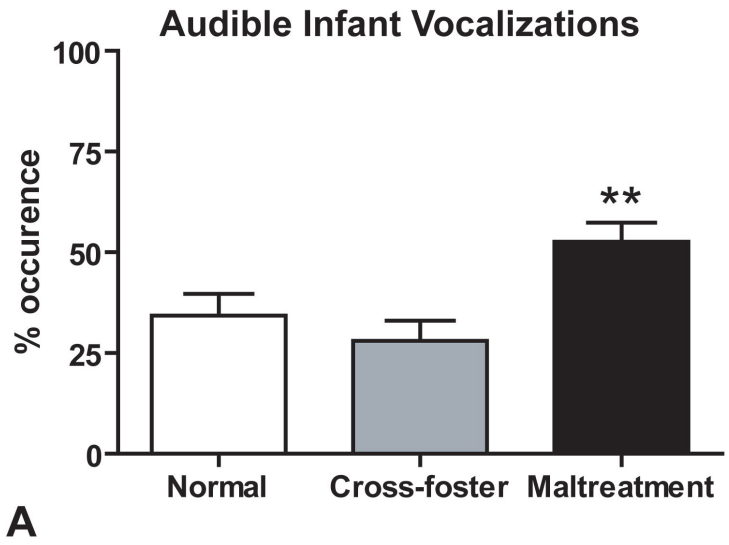


Figure 1 Observed maternal behavior across the three caregiving conditions. Dams in the normal maternal care (B) and cross-foster care (C) conditions were observed to have higher levels of nurturing care compared with the dams in the maltreatment (D) condition which had higher occurrences of aversive care. n=18-22 dams per group; error bars represent SEM.



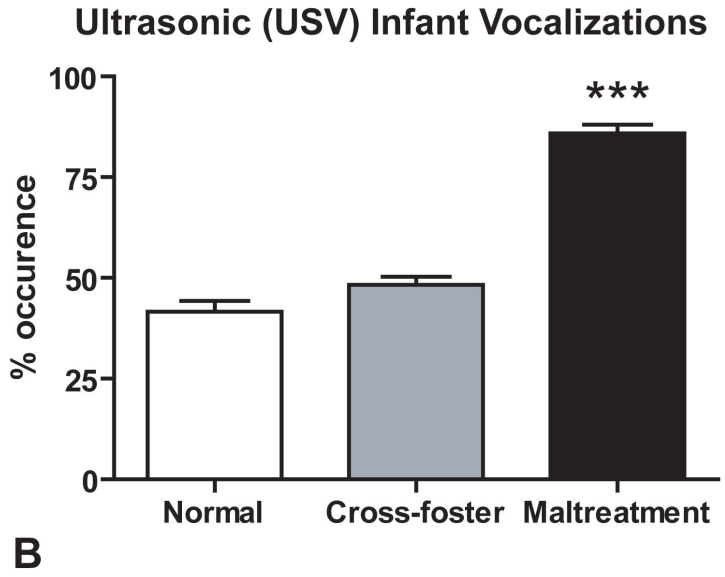


Figure 2 Infant audible and ultrasonic vocalizations in the three caregiving conditions. Infants in the maltreatment condition emitted significantly more (A) audible and (B) ultrasonic vocalizations compared to infants from the normal maternal care and cross-foster care conditions. **p<0.01, ***p<0.001, and error bars represent SEM.

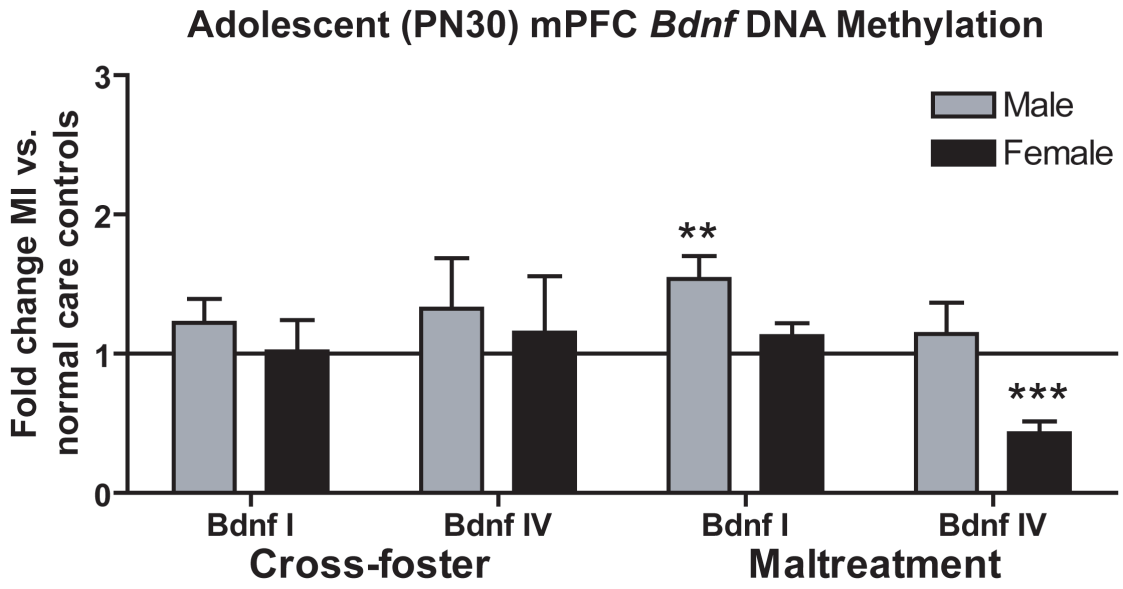


Figure 3 *Bdnf* DNA methylation across sex and maternal-care condition at exons I and IV in adolescent (PN30) rats. Maltreated males had a significant increase in DNA methylation at *Bdnf* exon I versus normal care controls, **p<0.01. Maltreated females had a significant decrease in DNA methylation at *Bdnf* exon IV compared with normal care controls, ***p<0.001. In each condition and sex, n=9-10 per group, and error bars represent SEM.

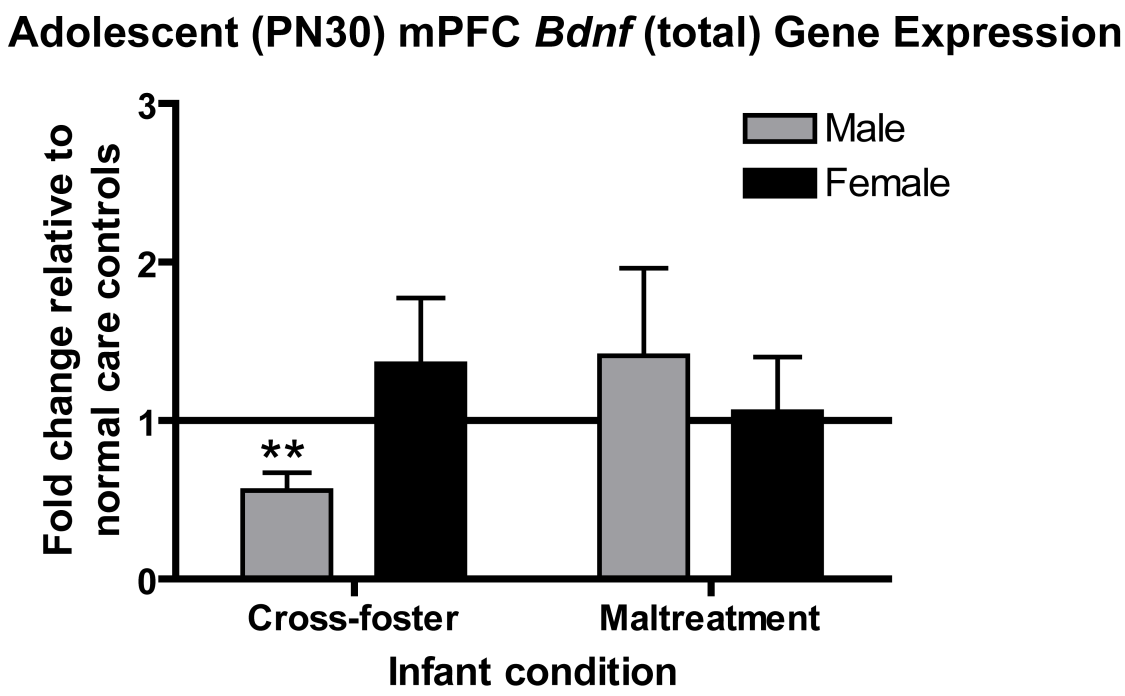


Figure 4 *Bdnf* mRNA gene expression differences across sex and maternal-care conditions. There was a decrease in *Bdnf* mRNA levels in cross-foster care males compared with normal care controls, **p<0.01. In the each condition and sex, n=7-10 per group, and error bars represent SEM.

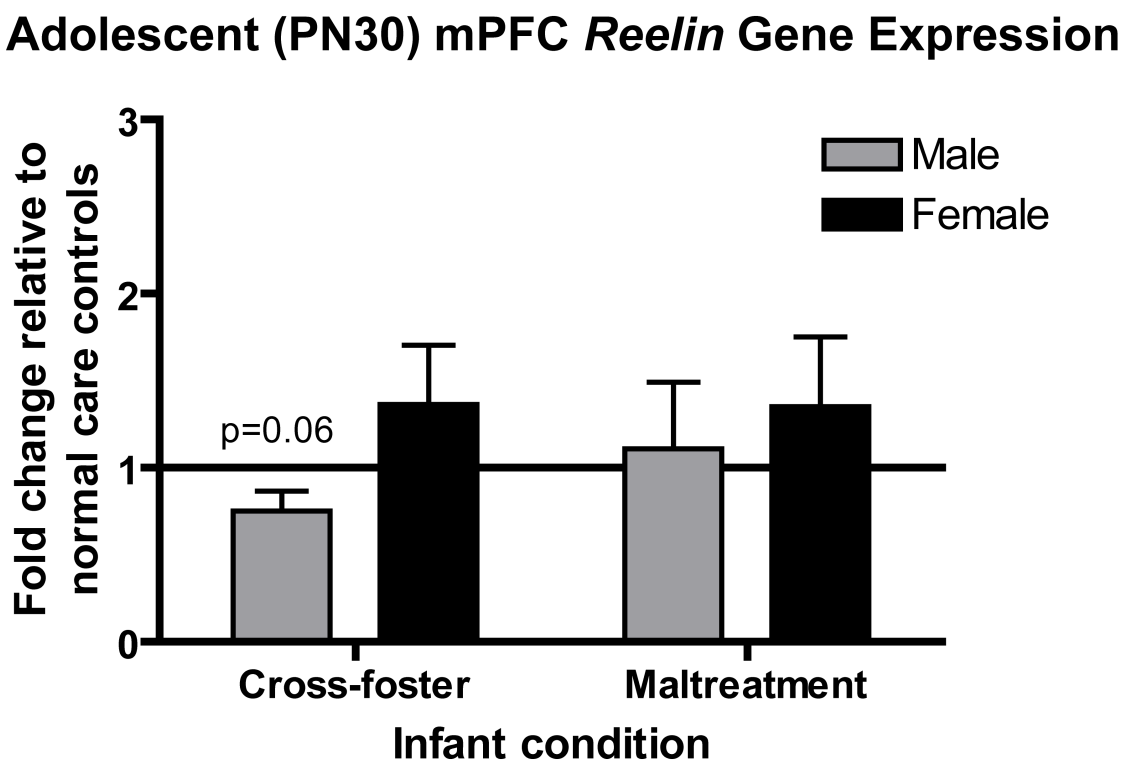


Figure 5 *Reelin* mRNA gene expression differences across sex and maternal-care condition in adolescent rats. There was a trending decrease in *Reelin* mRNA levels in cross-foster care males compared with normal care controls, p=0.06. In each condition and sex, n=7-10, and error bars represent SEM.