CHARACTERIZATION OF ADVERSITY-INDUCED PHENOTYPIC OUTCOMES AND PREVENTION OF ADVERSITY-INDUCED *BDNF* METHYLATION VIA PHARMACOLOGICAL MANIPULATION OF THE EPIGENOME

by

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A dissertation submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Psychology

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PREFACE

I, Tiffany Doherty, hold principle author status for the chapters comprising this dissertation. Each of these chapters have co-authors whose contribution greatly facilitated my dissertation research, as detailed below.

Chapter 1 consists of segments from a manuscript co-authored by my mentor, Dr. Tania Roth. This manuscript was published in *Development and Psychopathology* in a special themed issue in 2016.

Chapter 2 is co-authored by Dr. Tania Roth, Jenifer Blaze, & Samantha Keller. This manuscript was published in *Developmental Psychobiology* in 2017.

Chapter 3 is co-authored by Dr. Tania Roth, Johanna Chajes, Lauren Reich, & Hannah Duffy. This chapter was submitted in March 2019 to the *International Journal of Developmental Neuroscience* to be considered for publication in a special themed issue.

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ABSTRACT

The early postnatal environment, particularly in the context of the caregiving relationship, plays a major role in the establishment of typical and atypical behavioral and biological trajectories. Biological embedding of early adverse experiences disrupts function of the hypothalamic-pituitary-adrenal (HPA) axis, alters plasticity of the central nervous system (CNS), and increases risk of cardiac and enteric disease. Behaviorally, this often manifests as mood dysregulation, cognitive dysfunction, and physical health issues. Though the mechanisms underlying these outcomes remain unclear, shifting of the epigenetic landscape is a promising candidate. Previous work from our lab has uncovered adversity-induced methylation that rises at specific loci of the *Brain-derived Neurotrophic factor (Bdnf)* gene, which is a major player in both development and lifelong CNS plasticity. We hypothesize that these epigenetic changes are significant contributors to behavioral outcomes following early adversity. To test this hypothesis, our lab employs a rodent model of disrupted caregiving called the "Scarcity-Adversity Model of Low Nesting Resources". This model capitalizes on the principle of resource deprivation to induce poor caregiving behavior toward pups, allowing us to examine behavior and brain differences in nurtured versus maltreated subjects.

Chapter 1 of this dissertation introduces the reader to epigenetic modifications and their potential role in behavioral plasticity in response to environmental conditions. After a brief introduction to some specific epigenetic modifications and

enzymes, Chapter 1 focuses on literature on the developing epigenome in the context of postnatal adversity.

Chapter 2 details the characterization of behavioral outcomes in subjects exposed to our model of early caregiving adversity. Literature establishes a clear link between early adversity and atypical behavioral outcomes, but the form of early adversity varies widely across publications. To move forward in our investigation of the epigenetics of early stress, we first had to characterize the behavioral outcomes of our specific model of early adversity. We examined behavior in adolescence and adulthood following exposure to our model in infancy. We chose tasks to characterize depressive- and anxiety-like behavior as well as learning and memory performance, and discovered that maltreated animals exhibited age and sex-specific differences on various tasks. While adult maltreated subjects exhibited atypical behavioral patterns in the forced-swim task (arguably a measure of depressive-like behavior) and novel-object recognition (a learning and memory task), adult maltreated males exhibited significant deficits on a fear extinction task (a learning and memory task often used to study human disorders such as post-traumatic stress disorder, or PTSD). Characterizing the behavioral outcomes of our model allows us to move forward in our attempt to understand the molecular mechanisms that underlie them.

Chapter 3 of this dissertation is meant to establish our ability to manipulate the epigenome, something that will be required in order for us to understand how it contributes to behavioral phenotypes. A handful of drugs are known to affect the epigenome via actions on epigenetic enzymes. One of these drugs, sodium butyrate, is a histone deacetylase inhibitor. Histone deacetylases remove acetyl groups from the tails of histones, the proteins around which DNA wrap, making the charge of the

histone-DNA complex such that transcription is reduced. Thus, giving a histone deacetylase inhibitor such as sodium butyrate should create a more permissive transcriptional state. In addition, DNA methylation is affected via extensive crosstalk between histone modifications and methylation levels. We chose to use this drug to investigate whether we could prevent a previously reported, stable, long-term increase in methylation at *Bdnf* exon IX in the prefrontal cortex (PFC) following maltreatment in infancy. Initial results revealed that a 300 mg/kg dose was not sufficient to prevent this mark in the maltreated PFC. However, the mark was successfully prevented in the maltreated male PFC when the dose was increased to 400 mg/kg, a finding that did not hold in females. Examination of global levels of methylation and of another *Bdnf* exon not affected by stress at this time point revealed no differences between groups. Though this suggests a promising level of specificity, the mechanisms of that potential specificity are unclear. Taken together, these results allow us to move forward and investigate the behavioral impact of preventing a stable epigenetic mark of early adversity.

Chapter 4 summarizes the findings, limitations, and potential implications of the data contained in this dissertation.

The data presented in this document have helped characterize the behavioral implications of our model of early-adversity, have reinforced previously reported data on the epigenetic implications of our model, and have established our ability to manipulate adversity-induced methylation of *Bdnf* to better understand how it contributes to behavioral pathology. Further research is needed to move this work forward in females and to better understand the off-target effects of this drug treatment, objectives which are being addressed in ongoing projects in our lab.

Overall, the data contained here provide insights that will facilitate our attempts to understand how early adversity affects health across the lifespan, knowledge we hope will one day translate to both prevention and intervention efforts.

Chapter 1

INTRODUCTION TO EARLY LIFE ADVERSITY AND EPIGENETICS

The efforts of many neuroscientists are directed toward understanding the appreciable plasticity of the brain and behavior. In recent years, epigenetics has become a core of this focus as a prime mechanistic candidate for environmentally-driven behavioral modifications. Animal models have been instrumental in advancing our understanding of environmentally-driven changes to the epigenome in the developing and adult brain. This introduction focuses on such discoveries driven by adverse environments along with their associated behavioral outcomes. While much of the evidence discussed focuses on epigenetics within the central nervous system, several peripheral studies in humans who have experienced significant adversity are also highlighted. As we continue to unravel the link between epigenetics and phenotype, discerning the complexity and specificity of epigenetic changes brought about by environment disruption is an important step toward understanding optimal development and how to prevent or ameliorate behavioral deficits bred by disruptive environments.

1.1 Introduction

One of the most astonishing things about the CNS is its ability to adjust to the demands of an ever-changing environment. This plasticity allows for behavioral adaptations critical to survival, and the mechanisms behind it are of great interest to many in the fields of neuroscience, psychology, and psychiatry. These adaptations require environmentally-driven changes in gene expression in an organism from gestation through senescence, a feat we now know is made possible by dynamic modifications of DNA and its associated proteins, or chromatin. The idea that DNA codes for RNA, which codes for proteins, is the central dogma of molecular biology. Because proteins are essential to cell function, tight regulation of their synthesis is critical to homeostasis and adaptation. Epigenetics (literally: "on top of" genetics), a term first coined by Conrad Waddington (Waddington, 1957), allows this regulation via changes that bidirectionally control transcription and translation without changing the underlying DNA sequence. These mechanisms are highly responsive to environments and our experiences and thus are considered one major route by which environmental factors can catalyze changes in the nervous system, thereby altering behavior.

In this chapter, after a brief introduction to several epigenetic modifications, I examine evidence obtained from animal models on the nature of these modifications in response to environmental adversity. In addition, I briefly review evidence supporting the existence of these epigenetic modifications in humans. Lastly, I discuss

pharmacological and behavioral treatments and interventions known to affect the epigenome and behavior.

1.2 Epigenetics: The Fundamentals

DNA methylation (5mC). Methylation of DNA involves the addition of methyl groups to cytosines, typically at cytosine-guanine dinucleotides, and this modification generally results in a suppression of transcription due to impedance of transcription factors and the recruitment of repressor proteins (L. D. Moore, Le, & Fan, 2013). Methylation has also been found to occur in non-CG contexts (Lister et al., 2009; Ramsahoye et al., 2000). While the biological significance of non-CG methylation is not yet entirely clear, it has been associated with transcriptional suppression *in vitro* (Guo et al., 2014). DNA methyltransferases (DNMTs)**,** the enzymes that catalyze DNA methylation, are responsible for transferring methyl groups from *S*-adenosylmethionine to the C5 carbon of cytosines (L. D. Moore et al., 2013). Activity of demethylases such as GADD45β (Ma et al., 2009) appear responsible for removing methyl groups in an active fashion whereas TET1 has been implicated in passive demethylation via oxidation of methyl groups (Guo, Su, Zhong, Ming, & Song, 2011). DNMT1 mainly functions to maintain methylation patterns by targeting hemimethylated DNA during replication, whereas DNMT3a and DNMT3b are *de novo* methyltransferases responsible for establishing new patterns of methylation (Bestor, 2000). MECP2 is a protein that binds to DNA in a methyl-dependent manner, either recruiting corepressors such as HDACs and mSin3 (Nan et al., 1998) or coactivators such as CREB1 (Chahrour et al., 2008), thereby contributing to the

silencing or enhancement of gene expression in a context-dependent manner. See Figure 1.1 for a schematic of methylation and some of the enzymes involved.

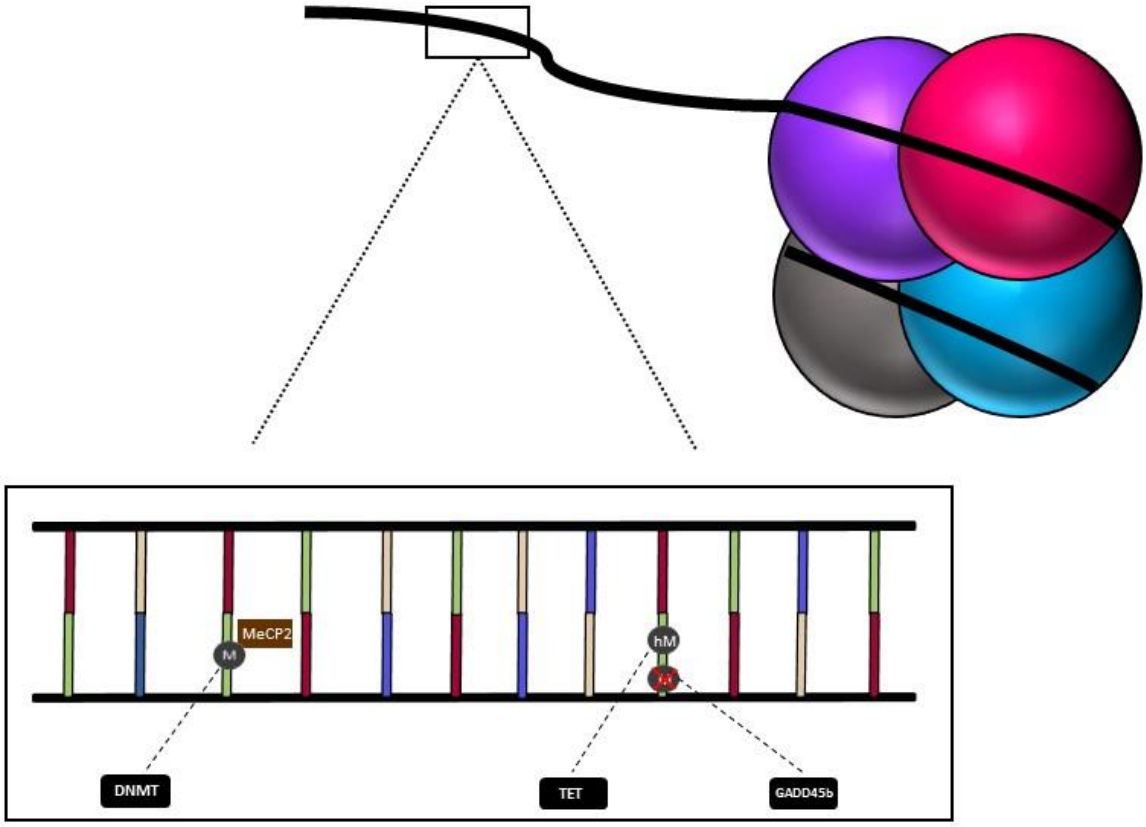


Figure 1.1 Schematic of DNA methylation and some of the enzymes involved in the addition or removal of methyl groups (Figure from Doherty and Roth, 2018).

Histone acetylation. Histone acetylation involves the addition of acetyl groups at lysine residues on the N-terminal tail of histone proteins, decreasing the affinity between the histone and DNA and thereby allowing a more permissive transcriptional state (Grunstein, 1997). This process is accomplished by histone acetyltransferases (HATs), which transfer the acetyl group from acetyl-CoA, and is reversed (i.e. the acetyl group removed) by histone deacetylases (HDACs) (De Ruijter, Van Gennip, Caron, Kemp, & van Kuilenburg, 2003).

Histone methylation. Histone methylation involves the addition of methyl groups at arginine and lysine residues on the N-terminal tail of histone proteins. Mono, di, or trimethylation can occur at lysine residues while only mono- or dimethylation can occur at arginine residues (Zhang & Reinberg, 2001). This process is accomplished by histone methyltransferases (HMTs) and is reversed (i.e. the methyl group removed) by histone demethylases. The direction of transcriptional regulation depends on the location and number of methyl groups. For example, monomethylation of histone 3 at lysine 36 (H3K36) and trimethylation of H3K4 (H3K4me3) are activational marks. Examples of repressive histone methylation marks are H3K9, H4K20, and H3K27me3.

1.3 Epigenetic Changes Driven by Postnatal Stress

One of the most widely studied environmental effectors of epigenetic change in rodents has been early-life stress (ELS) occurring during the early postnatal period.

Stress is studied in the rodent postnatal period commonly through examination of natural variations in (or manipulation of) the caregiving environment. Considering the programming effects of maternal behavior on HPA axis function and stress responsivity in offspring (S. Levine, 1994; Liu et al., 1997; Maccari et al., 2003) it is no surprise that this system has been a focal point in the search for mechanisms by which the early environment is able to shape biological and behavioral outcomes.

The HPA axis is a set of three structures (the hypothalamus, the anterior pituitary gland, and the adrenal glands) that plays a central role in stress responsivity (as reviewed in (Frodl & O'Keane, 2013; Herman & Cullinan, 1997; Leonard, 2005)). Briefly, corticotrophin-releasing hormone (CRH, also referred to as CRF) and arginine vasopressin (AVP) are released from the paraventricular nucleus (PVN) of the hypothalamus in response to stress. These hormones then trigger the production and release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary, which then goes on to trigger the production and release of corticosterone (cortisol in humans) from the adrenal glands (De Kloet, Vreugdenhil, Oitzl, & Joels, 1998).

Consistent with this focal point is one of the most well-known studies of environmentally-driven epigenetic changes, conducted in 2004 by Weaver and colleagues. Briefly, this study investigated natural variations in maternal care and found that adult male offspring of female rats that exhibited high levels of licking and grooming showed different epigenetic patterns at the glucocorticoid receptor (*GR*) gene than the male offspring of low licking and grooming females (Weaver et al., 2004). Offspring of mothers that exhibited lower levels of care were found to have high levels

of DNA methylation and less histone acetylation of hippocampal *GR* DNA, leading to less *GR* expression. This epigenetic, maternal care-induced reduction in GRs resulted in a lack of negative feedback in the HPA system and therefore to an exaggerated stress response in the offspring (when assessed in adulthood). Further demonstrated by this study was the reversibility of these outcomes either by moving pups to a positive caregiving environment (cross-fostering of pups to high-care mothers) or by altering epigenetic patterns via drug administration. These results demonstrated the capability of the early environment to shape the nervous system in such a way that behavior was altered throughout the life of the organism and more importantly, pinpointed epigenetics as a mechanism by which this incredible interaction was likely to occur.

Also focused on the HPA axis, a study by Murgatroyd and colleagues in 2009 found sustained epigenetic changes of the mouse *Avp* gene in response to maternal separation, another common inducer of early-life stress in rodents (Murgatroyd et al., 2009). Specifically, the authors discovered hypomethylated *Avp* DNA accompanied by less MeCP2 in the enhancer region and greater *Avp* mRNA in the PVN of offspring that had experienced maternal separation. These changes led to increased activity of the HPA axis as well as deficits in memory (as assessed by an inhibitory avoidance task) and stress coping (as assessed by a forced swim task). Further, administration of an AVP receptor antagonist proved able to partially or fully attenuate, respectively, these deficits. Additionally, higher mRNA levels of the precursor hormone for ACTH (pro-opiomelanocortin or *Pomc)* were seen in maternally separated animals, a change

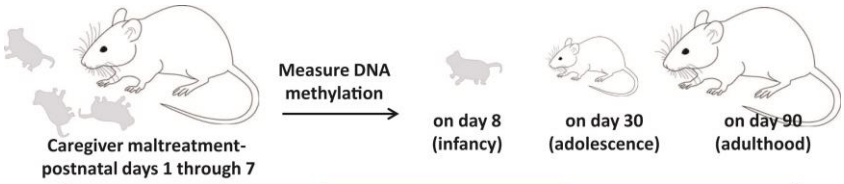
attributed to less methylation of the *Pomc* promoter region (Y. Wu, Patchev, Daniel, Almeida, & Spengler, 2014).

A more recent study along these lines demonstrated that while ELS resulted in hypermethylation of a region located a short distance from the *GR* promoter region in the PVN, an increase in *GR* mRNA was also seen (Bockmühl et al., 2015). Interestingly, ELS animals exposed to chronic stress in adulthood did not exhibit an increase in Crh when compared to controls, likely because of this *GR* upregulation. Considering here that higher methylation was not correlated with a decrease in gene expression, these data highlight that DNA methylation is not always synonymous with less gene expression and suggest that other factors (e.g. other epigenetic marks such as 5hmC) may be involved, interacting with 5mC to produce different outcomes. Altogether, data from these studies demonstrate long-term shaping of the HPA axis by the early environment that affects behavioral outcomes (whether adaptive or not) and pinpoints epigenetic changes as a mechanism of action.

Other studies have ventured outside of this stress response system to investigate whether there are ELS-induced epigenetic changes within other brain regions and genes of interest. Following maternal separation for 3 hours on each of the first 13 days of life, the Neurotensin receptor 1 gene *(Nt-r1*, a receptor for the neuropeptide neurotensin thought to play an important role in fear and anxiety) was found to be hypermethylated in the adult rodent amygdala (Toda et al., 2014). These rodents also displayed enhanced freezing behavior in a fear conditioning task. Given these results and the potential anxiolytic role of neurotensin (Saiz, Carrasco, & Hernanz, 1991; Shilling & Feifel,

2008), epigenetic changes to this gene may be a source of some of the behavioral outcomes associated with early-life stress.

Plasticity-related genes have also been a focus in behavioral epigenetics and have proven to be quite sensitive to early postnatal events. Using a rodent model wherein rat pups are briefly exposed (30 minutes) daily during the first week of life to an aversive form of caregiving (i.e. stepping on, dragging, roughly handling, actively avoiding), our lab has found changes in DNA methylation of plasticity-related genes in several different brain regions (Blaze & Roth, 2013; Roth, Matt, Chen, & Blaze, 2014). Several of these studies have focused on DNA associated with various exons of brain-derived neurotrophic factor (*Bdnf*), a gene critical to the processes of neural development and synaptic plasticity (Greenberg, Xu, Lu, & Hempstead, 2009). These studies underscore the complex nature of DNA methylation, finding ELS-induced changes in methylation that vary by exon examined, as well as by age, sex, and brain region (Figure 1.2).



Brain Region		DNA methylation patterns when adult		Were same patterns detected earlier in development?	
		bdnf IV	*bdnf* IX	*bdnf* IV	*bdnf* IX
PFC (whole)		higher	higher	no	yes
Brain Region	**Sex**	*bdnf* I	*bdnf* IV	*bdnf* I	*bdnf* IV
Medial PFC	male	lower	no change	no	yes
	female	lower	higher	no	no
Amygdala	male	lower	lower	no	no
	female	no change	lower	no	no
Dorsal Hippocampus	male	lower	lower	no	no
	female	no change	no change	no	no
Ventral Hippocampus	male	no change	higher	yes	no
	female	higher	no change	no	no

Figure 1.2 Schematic of *Bdnf* DNA methylation changes we have observed in the brain of infant (8 days old), adolescent (30 days old), and adult (90 days old) male and female rats exposed to our maltreatment regimen. Higher, lower, or no change refers to methylation levels in maltreated-rats in comparison to normal- (nurturing) care controls. Changes in methylation are for DNA associated with important regulatory or coding regions of the *Bdnf* gene (*Bdnf* I, IV, IX). Note we did not examine DNA associated with exon I for the prefrontal cortex (PFC) as a whole, nor did we examine DNA associated with exon IX in the mPFC, amygdala, or hippocampus. For the whole PFC study, male and female data were combined and sexes were not examined individually (Figure from Doherty and Roth, 2016, composed of data from Blaze et al., 2013.)

Following this type of ELS, rats of both sexes in adulthood, adolescence, and infancy exhibit specific changes in *Bdnf* DNA methylation in the (whole) (Roth, Lubin, Funk, & Sweatt, 2009) and medial prefrontal cortex (PFC and mPFC) and in the hippocampus (dorsal versus ventral) and amygdala (Blaze, Scheuing, & Roth, 2013; Tiffany S Doherty, Forster, & Roth, 2016; Roth et al., 2014). Further, altered patterns of histone acetylation at *Bdnf* exon IV were found in the mPFC of maltreated females (Blaze, Asok, & Roth, 2015). These latter results are especially interesting in light of recent findings describing downregulation of histone modifiers in the mPFC of maternally-separated rats (Pusalkar et al., 2015).

Also using our ELS paradigm, we have examined methylation of Reelin, another gene critical to brain development and synaptic plasticity (Curran & D'Arcangelo, 1998; Weeber et al., 2002), finding transiently low levels of mPFC methylation in maltreated females (i.e. this difference did not persist into adulthood) and a trend toward high methylation in maltreated males when adult (Blaze & Roth, 2013). Lastly, we have measured the expression of various epigenetic regulators occurring in response to this ELS model and found significant differences varying by sex and age in the mPFC of maltreated animals, with the strongest differences emerging in adulthood (Blaze & Roth, 2013). Altogether, our data indicate that amazingly brief environmental manipulations during the first postnatal week are sufficient to epigenetically modify genes, an effect that at some *Bdnf* loci (i.e. exon IX in the PFC) can be remarkably maintained throughout development and into adulthood. These data also help illustrate

the importance of incorporating a longitudinal approach in epigenetic studies as our results for other *Bdnf* loci (e.g. exon IV in the female PFC and male ventral hippocampus) indicate that maltreatment does not always translate into immediate epigenetic changes but results in changes that can evolve after an appreciable delay.

Altogether, data reviewed thus far tell us that ELS can alter the epigenome, suggesting that epigenetic changes may be partly responsible for the adverse outcomes associated with ELS such as changes in mood, cognition, and stress responsivity, (Cicchetti & Toth, 2005; Ivy et al., 2010; V. Lee & Hoaken, 2007), increased drug-seeking behavior (Dembo et al., 1989; Deminière et al., 1992; Huang et al., 2011), and psychiatric disorders (Cicchetti & Toth, 2005; Heim & Nemeroff, 2001; Kaffman & Meaney, 2007). Epigenetic changes may also be responsible for altered patterns of parenting, another realm of behavior significantly affected by ELS. It has been well established in humans that patterns of abuse are often perpetuated from one generation to the next (Haapasalo & Aaltonen, 1999; Ney, 1988; Zaidi, Knutson, & Mehm, 1989), and animal models have allowed for investigation of the molecular substrates of this perpetuation.

Female rodents receiving low levels of maternal care in infancy will in turn exhibit low levels of care toward their own pups (Francis, Diorio, Liu, & Meaney, 1999). From the early to mid-2000s, Michael Meaney's laboratory made a series of discoveries which have greatly contributed to our understanding of this phenomenon. Initially, this group reported low levels of oxytocin receptor binding in rodent mothers displaying low levels of care toward pups (Francis, Champagne, & Meaney, 2000), and

then that sensitivity to estrogen, which increased binding at these receptors in brain regions relevant to maternal behavior, was lower in the offspring of low-care mothers (F. Champagne, Diorio, Sharma, & Meaney, 2001). Further, they found lower levels of estrogen receptor *(ER)-α* mRNA in the medial preoptic area (MPOA) of low versus high-care females (F. Champagne, Weaver, Diorio, Sharma, & Meaney, 2003). Additionally, they reported an association between differential methylation of the *ER-α* promoter region in the MPOA between low- and high-care females (F. Champagne et al., 2006). Cross-fostering the offspring in these studies led to biological and behavioral outcomes resembling those of the caretaker, not the biological mother, suggesting that transmission of these patterns is nongenomic. Taken as a whole, these studies suggest that estrogen-induced oxytocin binding is a critical component of rodent maternal behavior and that DNA methylation may be the mechanism by which differences in this biological state, and therefore differences in maternal behavior, are passed from one generation to the next.

A recurring theme in many of the studies discussed above is sex differences in the epigenetic state and their associated behavioral outcomes. Perhaps not surprisingly, maternal behavior may play a significant role in these differences. Rodent mothers exhibit a different pattern of behavior toward infant male offspring than they do infant female offspring, with male pups receiving higher levels of maternal attention through behaviors such as licking and grooming (C. L. Moore & Morelli, 1979; Richmond & Sachs, 1984). When compared to mothers of all-female or mixed-sex litters, higher nurturing behavior (e.g. licking, active nursing, nest building) has been observed in

mothers of all-male litters (Alleva, Caprioli, & Laviola, 1989; Cirulli, Adriani, & Laviola, 1997). These differences affect offspring outcomes, likely via epigenetic pathways. For example, in male- or female-only (single-sex) litters the mu-opioid receptor-encoding gene *Oprm1* exhibits site-specific hypermethylation in the hippocampus (males and females) and nucleus accumbens (males only) (Hao, Huang, Nielsen, & Kosten, 2011). This receptor is thought to be involved in both attachment and reward (Kieffer & Gavériaux-Ruff, 2002; Matthes et al., 1996; Moles, Kieffer, & D'Amato, 2004; Nelson & Panksepp, 1998), making it an interesting target considering that reward-based behaviors such as drug use may be intimately related to disruptions in caregiver attachment (Kosten, Miserendino, & Kehoe, 2000). Indeed, endogenous opioids are released by the infant during caregiver interactions (Nelson & Panksepp, 1998; Roth & Sullivan, 2006; Weller & Feldman, 2003). Thus, it appears that the caregiving environment plays a programming role in the development of this system.

Using the same litter composition model (mixed-sex versus single-sex), methylation levels of *Nr3c1* (*GR* gene), *Egr1* (Early growth response protein 1; a transcription factor that may be involved in neuronal plasticity (Knapska & Kaczmarek, 2004)), and *Bdnf* were measured in the nucleus accumbens and hippocampus of offspring (Kosten, Huang, & Nielsen, 2014). In the nucleus accumbens, a number of cytosine sites in the *Nr3c1* promoter region exhibited higher methylation in female pups reared in single-sex litters. In male pups reared in single-sex litters, *Egr1* methylation was lower in this same brain region. *Bdnf* methylation was not significantly different between groups in either brain region. Given that we know *GR* gene methylation is

higher when maternal (nurturing) interactions are lacking, and that female pups receive less of this nurturing behavior when compared to male pups, these *Nr3c1* data appear consistent with one another. This study also assessed fear and anxiety behaviors on postnatal day 35 but found no significant effect of litter composition, making the results difficult to interpret at a phenotypic level. Given that many studies find changes in adult behavior following disruption of the early environment, it is reasonable to suggest that litter composition could affect these behaviors at a later time point. Overall, differences in the sex composition of a litter affect maternal behavior, behavior that is already differentially exhibited toward male and female pups. Through animal models we have come to understand that these factors alone (i.e. litter composition and male-directed maternal behavior) are environmental drivers of epigenetic change. Understanding their combined effect will help further elucidate the relationship between the early environment and epigenetically-mediated behavioral outcomes, specifically those of a sex-specific nature.

While the majority of studies I have discussed thus far have focused on specific genes, several have also zoomed out to investigate global activity of the epigenome. While there are some challenges in this approach (including costs and burdensome data sets), increasing evidence suggests that changes in genome-wide levels of methylation are associated with maladaptive behavioral outcomes (Anier et al., 2014; Kinnally et al., 2011). Understanding epigenetic activity on a broad scale is of course instrumental in understanding larger behavioral domains (i.e. a psychiatric disorder) and particularly

for treatment considering that current drug and behavior therapies will act on a global level.

Differential promoter methylation on a broad scale has been seen in the brain (PFC) and in T-cells (a component of the immune system) of maternal- versus peer-reared rhesus macaques (Provençal et al., 2012). Additionally, these groups showed differential levels of hydroxymethylation in the promoter region of numerous neurologically and psychologically relevant genes (Massart et al., 2014). While there is little research thus far on epigenetic regulation of the immune system following ELS, it is a promising regulatory candidate for the ELS-induced immune changes seen in both animals and humans (Miller et al., 2009; O'Mahony et al., 2009). Though no genome-wide effects were seen in the hippocampus in an earlier study by Brown and colleagues focusing on natural variations in maternal care (Brown, Weaver, Meaney, & Szyf, 2008), our lab recently found changes in global levels of methylation and hydroxymethylation in adolescent rats exposed to caregiver maltreatment. Specifically, maltreated males exhibited high 5-mC levels in the dorsal hippocampus and low 5-hmC levels in the amygdala (Tiffany S Doherty et al., 2016). This change in global 5-hmC may be of interest in particular as evidence suggests that this cytosine modification plays a pivotal role in behavioral adaptation (Li et al., 2014).

Many of the animal findings of epigenetic consequences associated with ELS have been translated to humans. Some of the most compelling results in this regard are those of a 2009 study from Patrick McGowan and colleagues. An investigation of postmortem hippocampal tissue revealed that in suicide victims with a history of child

abuse, *GR* promoter methylation and gene expression were significantly increased and decreased, respectively (McGowan et al., 2009). These results mirror those found in the low-care rodent offspring discussed previously. Infants of mothers with postpartum depression have been found to exhibit significant methylation of the *GR* gene as well, an effect that is attenuated with tactile stimulation (i.e. stroking) of the infant by its mother (Murgatroyd, Quinn, Sharp, Pickles, & Hill, 2015). Not unexpectedly, higher *GR* methylation is also seen in adolescents following acute social stress, a change accompanied by aberrant cortisol recovery (Van Der Knaap, Oldehinkel, Verhulst, Van Oort, & Riese, 2015). Astonishingly, one line of work is even showing far-reaching *GR* methylation changes (and stress responsivity) in Holocaust survivor offspring (Yehuda et al., 2014).

In addition, methylation of *Bdnf* (Perroud et al., 2013; Unternaehrer et al., 2015; Weder et al., 2014), the oxytocin receptor gene (*OXTR* (Unternaehrer et al., 2015), the serotonin transporter gene *(SLC6A4* (Beach, Brody, Todorov, Gunter, & Philibert, 2011; Vijayendran, Beach, Plume, Brody, & Philibert, 2012)) and *FKBP5* (Mehta et al., 2011; Weder et al., 2014) are associated with the type of parental care experienced in childhood. Of note, high levels of methylation in individuals who report experiencing child abuse and neglect mirror many of the brain observations we have found in our maltreated rats. In summary, data are consistent between animal models and humans, arguing that epigenetic changes are one route through which early postnatal environments could be yielding their behavioral effects.

1.4 Prevention and Intervention Research Regarding the Epigenome

Recently, much interest has been generated around epigenetic therapy. Drugs such as 5-aza-2'-deoxycytidine and zebularine (DNA methyltransferase inhibitors; DNMTi) primarily inhibit DNA methylation while others, such as sodium butyrate or valproic acid (histone deacetylase inhibitors; HDACi), primarily inhibit histone deacetylation. It is important to note that these drugs and their primary epigenetic modifications are not mutually exclusive. Rather, there is much cross talk between them. For example, DNA methylation alters histone marks through the recruitment of HDACs via MeCP2 (Rountree, Bachman, Herman, & Baylin, 2001), and histone deacetylase inhibitors reduce methylation, likely via downregulation of DNMT1 (S. Sarkar et al., 2011) and upregulation of DNA demethylase (Detich, Bovenzi, & Szyf, 2003).

Epigenetic drugs in various animal models have proven to be effective in ameliorating behavioral deficits by reversing or preventing deleterious epigenetic marks. For example, in the well-known and previously discussed study conducted by Weaver and colleagues, central administration of a HDACi ameliorated both the epigenetic changes and behavioral deficits found in low-care offspring (Weaver et al., 2004). Similarly, Roth and colleagues found that administration of a DNMTi successfully reversed *Bdnf* methylation and gene expression changes found in adult rats that had experienced maltreatment in infancy (Roth et al., 2009). In 2012, Kao and colleagues reported that administration of a HDACi prior to maternal separation prevented separation-induced histone methylation (Kao et al., 2012). These histone

marks were associated with a decrease in fear-potentiated startle in female offspring in adulthood, a behavioral deficit that was prevented with HDACi administration.

Given that chromatin modifications are often seen in psychiatric disorders (Klengel et al., 2013; Matrisciano et al., 2013) and specific HDACs have been implicated in disorders such as depression (Tsankova et al., 2006) and schizophrenia (Sharma, Grayson, & Gavin, 2008), drugs that can target these modifications seem to hold promise for psychiatric intervention. Indeed, co-administration of the HDACi sodium butyrate with the antidepressant fluoxetine (which also affects histone modifications (Cassel et al., 2006; Hunter, McCarthy, Milne, Pfaff, & McEwen, 2009)) reduces depressive-like behavior in mice (Schroeder, Lin, Crusio, & Akbarian, 2007). Administration of DNMT inhibitors showed similar effects in rats (Sales et al., 2011). The HDACi valproic acid is able to attenuate schizophrenic-like behaviors induced by methionine in mice (Tremolizzo et al., 2005). Data such as these are particularly relevant to the focus of this review as early life stress is often a precipitating factor for many psychiatric disorders (Cicchetti & Toth, 2005; Jovanovic et al., 2009).

Lastly, while many of the epigenetic drugs used in animal models are not yet approved for humans (outside of cancer treatment), many psychotropic drugs prescribed to humans affect epigenetic activity (some of which are also used in animal models, discussed in previous sections). For example, the antipsychotic drug haloperidol affects DNA methylation (Shimabukuro, Jinno, Fuke, & Okazaki, 2006) and some histone modifications (Bertran-Gonzalez et al., 2008) while MAO inhibitors affect both mono- and dimethylation of histones (M. G. Lee, Wynder, Schmidt, McCafferty, &

Shiekhattar, 2006). Behavioral therapies are also proving to be very effective at reshaping the epigenome. Children diagnosed with anxiety disorders who respond well to cognitive behavioral therapy exhibit higher methylation of the stress- and depression-related *SLC6A4* gene (Roberts et al., 2014). Also, as previously discussed, the epigenome of subjects diagnosed with borderline personality disorder who exhibited a positive response to behavioral therapy exhibited changes to previously hypermethylated regions of DNA as well (Perroud et al., 2013).

Other environmental alterations that have been found to be impactful on the human epigenome include exercise and practices such as tai chi. Regarding the former: genome-wide methylation patterns in adipose tissue of low-activity individuals exhibited significant changes in response to a 6-month exercise intervention (Rönn et al., 2013). In regard to the latter: females practicing tai chi for at least three years exhibit differential methylation patterns when compared to age-matched controls, with methylation patterns opposing those generally associated with increased age (Ren et al., 2012). Altogether, the data reviewed in this introduction demonstrate the malleability of the epigenome to not just adversity, but positive experiences and therapeutic interventions.

1.5 Conclusions

The ability of the developing organism to change the central nervous system in such a way that its behavior matches the environment in which it will likely be required

to survive is a highly adaptive characteristic. Evidence from animal models suggests that epigenetic mechanisms possess the responsiveness and plasticity necessary to make this adaptation possible. Indeed, the tissue specificity and sexual dimorphism of environmentally induced epigenetic modifications suggest that they are functionally significant, and behavioral data continue to support this notion. Additionally, data thus far demonstrate that the epigenome is responsive to behavioral therapies, pharmacological manipulations, and lifestyle factors such as diet and enrichment, making it a promising target for intervention. Most promising, perhaps, is that we are beginning to see these data replicated in humans.

Epigenetic modifications mark experiences such that transcriptional regimes are altered in a cascade that begins in the nucleus and expands to shape behavioral outcomes. Some of these marks and their associated outcomes are favorable to the organism while others are not. One very important task for our field is to parse apart these differences in enough detail that we can use epigenetic information to inform diagnoses as well as targeted treatments to produce the most favorable outcomes. Inherent in this process will be the need for carefully-designed longitudinal studies, which will allow the elucidation of the developmental trajectories of epigenetic changes and behavior. Further, to better inform diagnoses, studies will need to incorporate the use of multiple biospecimens, an approach easily achieved in rodent models but to date has been rarely employed. Finally, in terms of treatment, one of the major challenges for epigenetic therapy is target specificity of drugs (an issue that is true of most drugs used in psychiatry). Animal model and *in vitro* work is already pioneering the way for

this, using targeted epigenetic editing, such as TALEs fused with chromatin modifying enzymes (Bernstein, Le Lay, Ruano, & Kaestner, 2015; Heller et al., 2014; Konermann et al., 2013; Maeder et al., 2013; Sanjana et al., 2012), to alter cytosine methylation at specific gene loci. Strategies to target specific epigenetic changes in humans may be coming over the horizon.

Chapter 2

BEHAVIORAL TRAJECTORIES ACROSS THE LIFESPAN FOLLOWING EXPOSURE TO ADVERSITY IN INFANCY

Early-life adversity is known to disrupt behavioral trajectories and many rodent models have been developed to characterize these stress-induced outcomes. One example is the scarcity-adversity model of low nesting resources. This model employs resource scarcity (i.e., low nesting materials) to elicit adverse caregiving conditions (including maltreatment) toward rodent neonates. Our lab utilizes a version of this model wherein caregiving exposures occur outside the home cage during the first postnatal week. The aim of this study was to determine adolescent and adult phenotypic outcomes associated with this model, including assessment of depressive- and anxiety-like behaviors and performance in different cognitive domains. Exposure to adverse caregiving had no effect on adolescent behavioral performance whereas exposure significantly impaired adult behavioral performance. Further, adult behavioral assays revealed substantial differences between sexes. Overall, data demonstrate the ability of repeated exposure to brief bouts of maltreatment outside the home cage in infancy to impact the development of several behavioral domains later in life.

2.1 Introduction

Adversity early in development is known to disrupt typical behavioral trajectories in both rodents (Anier et al., 2014; Kundakovic, Lim, Gudsnuk, & Champagne, 2013; Pan, Fleming, Lawson, Jenkins, & McGowan, 2014) and humans (Agid et al., 1999; Cicchetti & Toth, 1995; McCrory, De Brito, & Viding, 2012). In rodents this includes increasing depressive- (Lippmann, Bress, Nemeroff, Plotsky, & Monteggia, 2007) and anxiety-like (Sarro, Sullivan, & Barr, 2014) behaviors as well as altering cognitive abilities (Sousa et al., 2014). Early adverse experiences often occur within the context of the caregiving relationship and several rodent models have been developed to study this type of adversity. Some examples include maternal separation (Biggio et al., 2014; Cotella, Mestres Lascano, Franchioni, Levin, & Suárez, 2013; Huot, Plotsky, Lenox, & McNamara, 2002; Zalosnik, Pollano, Trujillo, Suarez, & Durando, 2014), fragmented maternal care (Gilles, Schultz, & Baram, 1996; Ivy, Brunson, Sandman, & Baram, 2008; Molet et al., 2016; Rice, Sandman, Lenjavi, & Baram, 2008), naturally occurring low levels of maternal care (Beery, McEwen, MacIsaac, Francis, & Kobor, 2016; F. Champagne et al., 2004; Weaver et al., 2004), and resource scarcity with adverse caregiving (Raineki, Cortés, Belnoue, & Sullivan, 2012; Raineki et al., 2015; Roth & Sullivan, 2005). Our lab uses a form of the latter model termed the scarcity-adversity model of low nesting resources wherein pup exposures occur outside the home cage with dams placed in a novel environment and given insufficient nesting materials. This results in disrupted maternal care such that

adverse behavior directed toward pups (i.e. dragging, dropping, stepping on, roughly handling, or actively avoiding pups) is significantly increased when compared to pups in the home cage or in a control condition where nesting resources are plentiful (Tiffany S Doherty et al., 2016; Roth et al., 2009; Roth et al., 2014).

These different early stress models often produce different behavioral outcomes. For example, offspring of naturally low-licking and grooming dams exhibit significantly increased levels of anxiety-like behavior in the open field test when compared to offspring of naturally high-licking and grooming dams (Caldji et al., 1998). In contrast, adult mice exposed to fragmented maternal care in infancy exhibit no significant behavioral differences in this same test when compared to controls (Rice et al., 2008). Behavioral outcomes can also differ within the same model, presumably based on variations in model parameters. For example, maternal separation has been shown to both diminish (Stevenson, Spicer, Mason, & Marsden, 2009) and enhance (Toda et al., 2014) conditioned fear in adult male rats (using 6-hour and 3-hour separations, respectively). These variations across and within models indicate that the phenotypic impact of each model (and variation therein) should be examined individually.

The aim of this study was to characterize phenotypic outcomes in the scarcity-adversity model of low nesting resources outside the home cage. Our lab has previously identified the impact of these brief and repeated exposures on one realm of behavior, maternal behavior (Roth et al., 2009), as well as the epigenome in several brain areas of adolescent and adult animals (Blaze et al., 2015; Blaze & Roth, 2013;

Blaze et al., 2013; Roth et al., 2014). Here we explore the development of several other behavioral domains following exposure to our model.

2.2 Methods

All procedures were conducted by the standards and with the approval of the University of Delaware Animal Care and Use committee.

2.2.1 Subjects and Caregiving Manipulations

Subjects consisted of male and female Long-Evans rat pups bred in-house. Dams were housed in polypropylene cages with wood shavings and kept in temperature controlled room on a 12-hour light/dark cycle. Food and water were available ad libitum. On postnatal (PN) day 1 (the day after birth) litters were culled to 5-6 males and 5-6 females.

Caregiving manipulations were conducted as previously reported by our lab (Blaze et al., 2013; Tiffany S Doherty et al., 2016; Roth et al., 2009; Roth et al., 2014). Pups were exposed to one of three conditions (maltreatment, cross-foster, and normal-care) 30 minutes daily for the first seven days of life. Pups in the maltreatment condition were exposed to a novel environment with a lactating dam (matched in diet and postpartum age to the birth mother of the experimental pups) given insufficient nesting resources and no time to habituate to her surroundings. Pups in the cross-foster condition were exposed to a novel environment with a lactating dam (likewise

matched in diet and postpartum age) given ample nesting materials and ample time to habituate to her environment (approximately 1 hour). Pups in the normal-care condition were simply weighed and returned to their biological mother in the home cage. All sessions were recorded and scored by two trained observers who marked each occurrence (in 5-minute time bins) of both nurturing caregiving behaviors (nursing, licking and grooming) and adverse caregiving behaviors (stepping on, dropping, dragging, roughly handling, or actively avoiding pups). An average of the two observer's scores was then taken for statistical analysis. Audible and ultrasonic (40 kHz) pup vocalizations were also recorded during each session. Two trained individuals scored each audio file and marked the occurrence of vocalizations (in 1-minute time bins). An average of their scores was then taken for statistical analysis.

Following the last exposure on PN7 pups were left undisturbed in the home cage until weaning between PN21-23. At this time they were pair-housed with a same-sex, same-condition littermate until behavioral tests at PN30 or PN90.

2.2.2 Adolescent and Adult Behavioral Testing

Subjects were tested in adolescence and adulthood (PN30 and PN90, respectively) and no more than 2 males and 2 females from a litter were assigned to an experimental condition in order to avoid litter effects. One cohort was used for adolescent open field, novel object recognition, and forced swim data (tests run in that order). A second cohort was used for adult data on the same tasks. A third cohort was

used for adolescent fear conditioning. A fourth cohort was used for adolescent novelty-suppressed feeding, marble burying, and sucrose preference (run in that order on consecutive days). A fifth cohort was used for adult novelty-suppressed feeding, marble burying, sucrose preference, and fear conditioning (run in that order on consecutive days). See Figure 2.1 for cohort layout. Subject numbers for all behavioral tests are provided in corresponding figure legends.

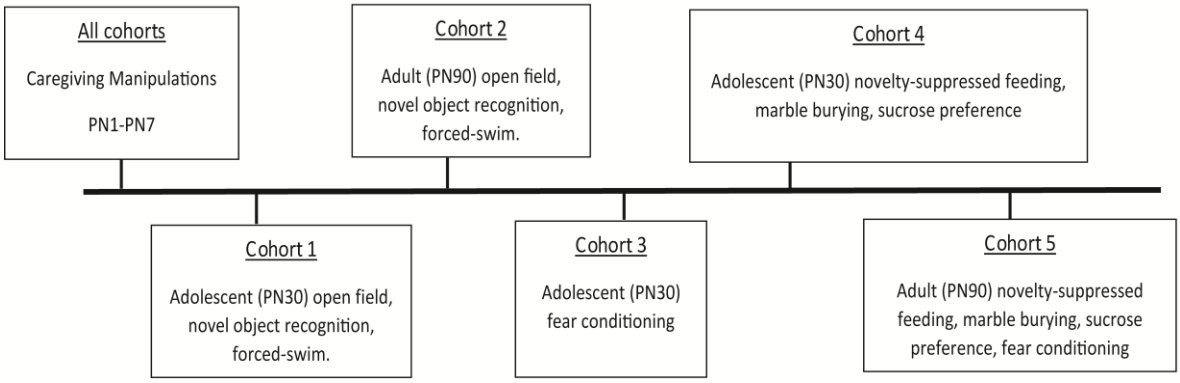


Figure 2.1 Depiction of the tasks assigned to each cohort used as well as the age at which they were assigned.

Sucrose Preference. Subjects were exposed to a 2-day sucrose preference procedure (adapted from (Posillico & Schwarz, 2016)). Rats were exposed during two consecutive sessions to two water bottles, one filled with tap water and the other with 1% sucrose water. Each bottle was weighed (in grams) before and after testing to determine consumption. Orientation of the bottles was switched between testing days to control for place preference. Day 1 was considered a habituation session to the two-bottle choice arrangement. A sucrose preference score was then derived from data obtained on day 2 by dividing the average weight in grams of sucrose consumed by the average weight in grams of total liquid consumed, multiplied by 100.

Forced swim test. Rats were exposed to the habituation phase of the Porsolt forced swim test (adapted from (Castagné, Moser, Roux, & Porsolt, 2011) in which they were placed in ~9 inches of room temperature (~25^{o}C) water in 10.75x11.75 inch (adolescent) or 19x11.5 inch (adult) buckets for 15 minutes. Rats were then thoroughly dried with a microfiber cloth and placed in a warm cage over a heating pad until dry. Twenty-four hours later, rats were again placed in the forced swim chamber for 5 minutes and time spent immobile (i.e., only making movements necessary to keep head above water) and latency to go immobile were scored by trained observers.

Novelty-suppressed feeding task. Rats were exposed to a novelty-suppressed feeding task (adapted from (Merali, Levac, & Anisman, 2003)). Rats were exposed for ten minutes to a familiar food reward (an almond slice) in an 8.5x15 inch (adolescent) or

14x33 inch (adult) novel, open arena. All testing was performed in red light with white noise (approximately 60 dB). Prior to testing day, subjects were familiarized with the almond slice via two home-cage exposures, one in the early morning and one in the late evening. The food reward was presented in the home cage in the same manner in which it was to be encountered in the arena (i.e. with the almond slice placed in the middle of a clear plastic petri dish). All animals began the test from the same point in the arena and latency to consume the food reward was recorded. The arena was cleaned thoroughly with ethanol between each subject.

Marble burying. Rats were exposed to a marble burying task (adapted from (Pandey, Yadav, Mahesh, & Rajkumar, 2009)). Rats were placed in individual polypropylene cages (20 cm x 46 cm x 23 cm) containing 5cm of clean bedding upon which ten clean marbles (15 mm diameter) were placed, evenly spaced throughout the cage. Subjects were then recorded for ten minutes and removed. Buried, partially buried, and unburied marbles were then counted and recorded.

Open field test. Rats were exposed to an open field test (adapted from (Arakawa, 2003)). All testing was performed in red light with white noise (approximately 60 dB). Subjects were handled (picked up and sat on experimenter's arm for two minutes) for two days prior to testing. On test day, rats were placed in an 8.5x15 inch (adolescent) or 14x33 inch (adult) circular open field for 10 minutes. Line crosses and time spent in the center of the field as well as number of entries into the center were video

recorded and scored by trained observers. The open field apparatus was cleaned thoroughly with ethanol between each subject.

Fear Conditioning. Fear conditioning, extinction, and retention testing protocols were performed in adolescent (adapted from (J. H. Kim, Li, & Richardson, 2010)) and adult (Chang et al., 2009) animals. Fear conditioning was performed in context A and involved three (adolescents) or five (adults) conditioned stimulus (CS)-unconditioned stimulus (US) pairings. The CS was a tone (2 kHz, 10 s, 80 dB) which co-terminated with the US foot shock (1 mA, 1s). Extinction training was conducted 24 hours later in context B and comprised 30 CS-only presentations. Extinction retention testing was conducted in context B (i.e. the extinction training context) 24 hours after extinction training and was comprised of five CS-only presentations. Contexts were made distinct by manipulating sensory cues (changing the floor from metal bars to smooth plastic, covering walls with checkered inserts, and changing the odor from ethanol to vanilla between contexts A and B). After every session, each apparatus was cleaned thoroughly with ethanol on day one or quatricide on day 2 (differed cleaning agents to maintain distinct odors between contexts). All behavioral sessions employed a 120s baseline period and 60s inter-trial intervals (ITI). Cameras located on the ceiling of the boxes recorded behavioral videos, which were scored in real-time for freezing behavior using ANY-maze software (Stoelting Inc.). Freezing during the CS presentation and the following ITI were blocked into one trial and converted into percentages for statistical analyses.

Novel object recognition (NOR). Rats were exposed to a novel object recognition task (adapted from (Oliveira, Hawk, Abel, & Havekes, 2010)). Following habituation to the chamber, subjects were exposed to two of the same objects (PN30: orange 50 ml conical tubes or black binder clips; PN90: orange 50 ml conical tubes or yellow measuring cups) secured in the open-field chamber (8.5x15 inch for adolescents or 14x33 inch for adults) for 15 minutes. Twenty-four hours later subjects were returned to the testing chamber with one of the objects from the previous day and one novel object for 15 minutes. The testing apparatus was thoroughly cleaned with ethanol between subjects. Video was recorded and trained observers scored time spent interacting with each object. These values were then used to obtain an exploration ratio (time spent exploring novel object/time spent exploring both).

2.2.3 Statistical Analyses

Two-way ANOVAs and when necessary, Bonferroni post-hoc tests were used to analyze caregiving behaviors (factors: caregiving behavior and infant condition), sucrose preference, marble burying, novelty-suppressed feeding, and open-field performance (factors: infant condition and sex). For fear conditioning analysis, freezing during the CS presentation and the following ITI were blocked into one trial and converted into percentages for statistical analyses. All behavioral data were subjected to an infant condition (NMC, MAL, CFC) x sex (male vs. female) x trial (1-

n) factor design. Analysis of variance (ANOVA) was performed to analyze freezing data. T-tests with Bonferroni corrections were applied when necessary. Finally, as is typical for analyzing novel-object recognition performance (Dix & Aggleton, 1999; Ramsaran, Westbrook, & Stanton, 2016), exploration ratios of each group were compared to chance performance (50%) using one-sample *t*-tests.

2.3 Results

2.3.1 Caregiving Manipulations

Assessment of caregiving behavior with a two-way ANOVA revealed a main effect of caregiving behavior ($F_{1,98} = 219.8$, $p<0.001$) and a behavior X infant condition interaction ($F_{2,98} = 57.56$, $p<0.001$). Infants exposed to normal- and cross-foster care experienced significantly higher levels of nurturing care and significantly lower levels of adverse care when compared to infants exposed to the maltreatment condition (p's<0.001). Adverse and nurturing behaviors are illustrated in Figure 2.2 for each condition. One-way ANOVAs revealed a significant effect of infant condition on the level of hovering/nursing ($F_{2,49} = 10.94$, $p<0.001$), stepping on pups ($F_{2,49} = 21.44$, $p<0.001$), dropping pups ($F_{2,49} = 4.376$, $p<0.05$), dragging pups ($F_{2,49} = 7.941$, $p<.01$), actively avoiding pups ($F_{2,49} = 15.46$, $p<0.001$), and roughly handling pups ($F_{2,49} = 9.023$, $p<.001$). No significant differences in licking/grooming behavior were found between groups ($p=0.244$). Bonferroni post-hoc tests revealed that when compared to normal and cross-foster care controls, maltreated pups experienced

significantly lower levels of hovering/nursing (p's<0.01) and higher levels of stepping (p's<0.001) and avoidance (p's<0.001) during manipulations. Maltreated pups were also dropped significantly more than cross-foster care pups ($p=0.0268$) and dragged (typically while nipple attached) significantly more than pups in the normal care condition ($p=0.0007$). Post-hoc analysis also revealed that pups in the cross-foster care condition experienced significantly lower levels of rough handling by the dam during experimental manipulations than both normal-care and maltreated pups (p's<0.05).

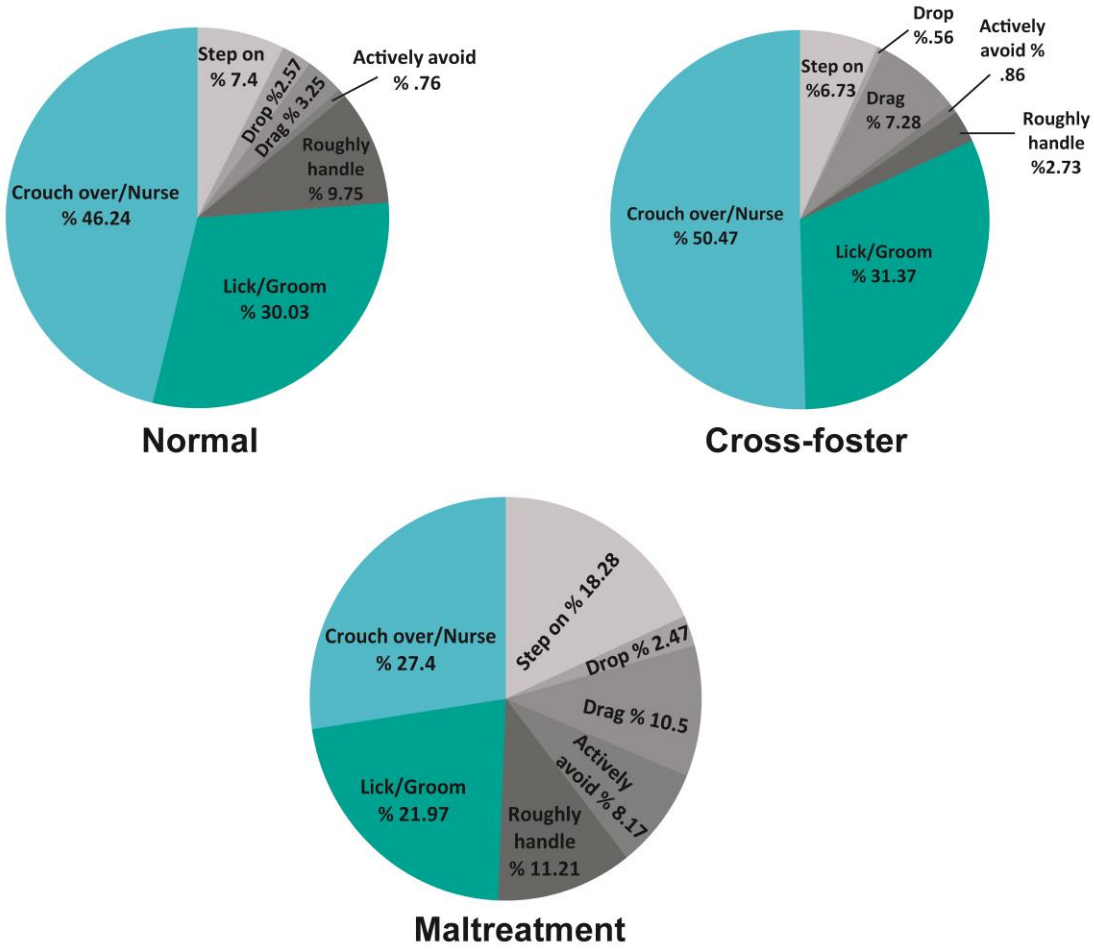


Figure 2.2 Distribution of nurturing and adverse behaviors exhibited by dams in each treatment group; n= 17-18 dams per group.

Additionally, levels of audible (mean percent occurrence in maltreated animals = 50.98, SEM = 3.62; mean percent occurrence in cross-fostered animals = 38.22, SEM = 4.057; mean percent occurrence in normal-care animals = 39.80, SEM = 3.529) and ultrasonic (mean percent occurrence in maltreated animals = 70.53, SEM = 5.650; mean percent occurrence in cross-fostered animals = 46.59, SEM = 5.122; mean percent occurrence in normal-care animals = 67.03, SEM = 5.317) vocalizations emitted by pups (see table 1) during caregiving manipulations were comparable to those previously reported by our lab (Blaze et al., 2013; Roth et al., 2014).

Pup Vocalizations	*F*	*p*	Bonferroni adjusted *p*	Mean	SEM
Audible	3.375	0.0427	*non-significant*	NMC: 39.80	3.529
				CFC: 38.22	4.057
				MAL: 50.98	3.62
Ultrasonic	5.805	0.0055	NMC vs CFC = 0.0292 CFC vs MAL = 0.0083	NMC: 67.03	5.317
				CFC: 46.59	5.122
				MAL: 70.53	5.65

Table 1. Means and statistical values from analyses of audible and ultrasonic vocalizations

2.3.2 Behavioral Assays

Infant manipulations had no effect on adolescent outcomes in any of the tasks used here to assess behavior (see table 2 for statistics). For adults however behavioral performance in many of these tasks was altered.

PN30 behavioral tasks	Interaction	Infant condition	Sex
Sucrose preference	$F_{2,22}$=0.7679, p =0.4760	$F_{2,22}$=0.1060, p =0.8999	$F_{1,22}$=0.1478, p =0.7043
Forced swim time spent immobile	$F_{2,66}$=0.1.544, p =0.2212	$F_{2,66}$=0.01239, p =0.9877	$F_{1,66}$=3.167, p =0.0797
Forced swim latency to immobility	$F_{2,66}$=1.636, p=0.2024	$F_{2,66}$=1.255, p=0.2916	$F_{1,66}$=1.303, p=0.2578
Marble burying (completely buried)	$F_{2,59}$=0.06443, p =0.9377	$F_{2,59}$=0.1860, p =0.8307	$F_{1,59}$=0.004005, p =0.9498
Open-field center entries	$F_{2,68}$=0.6691, p =0.5155	$F_{2,68}$=1.935, p =0.1523	$F_{1,68}$=0.1984, p =0.6574
Open-field time in center	$F_{2,68}$=1.531, p =0.2236	$F_{2,68}$=0.6686, p =0.5158	$F_{1,68}$=0.2594, p =0.6122
Novelty-suppressed feeding	$F_{2,60}$=1.291, p =0.2825	$F_{2,60}$=2.472, p =0.0929	$F_{1,60}$=1.371, p =0.2463
	Normal-care	**Cross-foster care**	**Maltreated**
Novel object recognition (versus chance at 0.5)	♂ t=4.750, p =0.0177 ♀ t=4.266, p =0.0053	♂ t=8.918, p =0.0123 ♀ t=6.219, p =0.0016	♂ t=4.694, p =0.0054 ♀ t=4.848, p =0.0029
Fear Behavior	**Fear conditioning**	**Fear extinction**	**Extinction retention**
Sex X infant condition	$F_{2,59}$=2.558, p=0.086	$F_{2,59}$=1.612, p =0.208	$F_{2,59}$=1.956, p=0.15
Trial X sex X infant condition	$F_{6,177}$=0.525, p=0.766	$F_{30,885}$=0.788, p =0.706	$F_{10,295}$=1.2.83, p=0.246
Sex	$F_{1,59}$=0.004, p=0.949	$F_{1,59}$=0.770, p =0.384	$F_{1,59}$=0.194, p=0.661
Infant condition	$F_{2,59}$=1.281, p =0.285	$F_{2,59}$=0.059, p=0.943	$F_{2,59}$=0.176, p =0.839
Trial	$F_{3,177}$=296.044, p <.001	$F_{15,885}$=21.855, p <.001	$F_{5,295}$=25.155, p <.001

Table 2. Statistical values from analyses of each adolescent behavioral task.

Sucrose preference and forced swim behavior were used to characterize depressive-like adult behaviors (Figure 2.3). For sucrose preference (Figure 2.3A), a two-way ANOVA revealed no significant differences between groups on sucrose preference scores for either habituation (day 1) or testing (day 2) (all p's > 0.05 for sex, caregiving condition, and interaction). While there were no significant effects of condition or sex on total time spent immobile in the forced swim test, a two-way ANOVA did reveal a significant effect of caregiving condition on latency to go immobile ($F_{(2,54)}$=4.850, $p<0.05$, Figure 2.3B). Bonferroni post-hoc tests indicate a longer latency to immobility in maltreated subjects when compared to their normal-care counterparts ($p = 0.0098$).

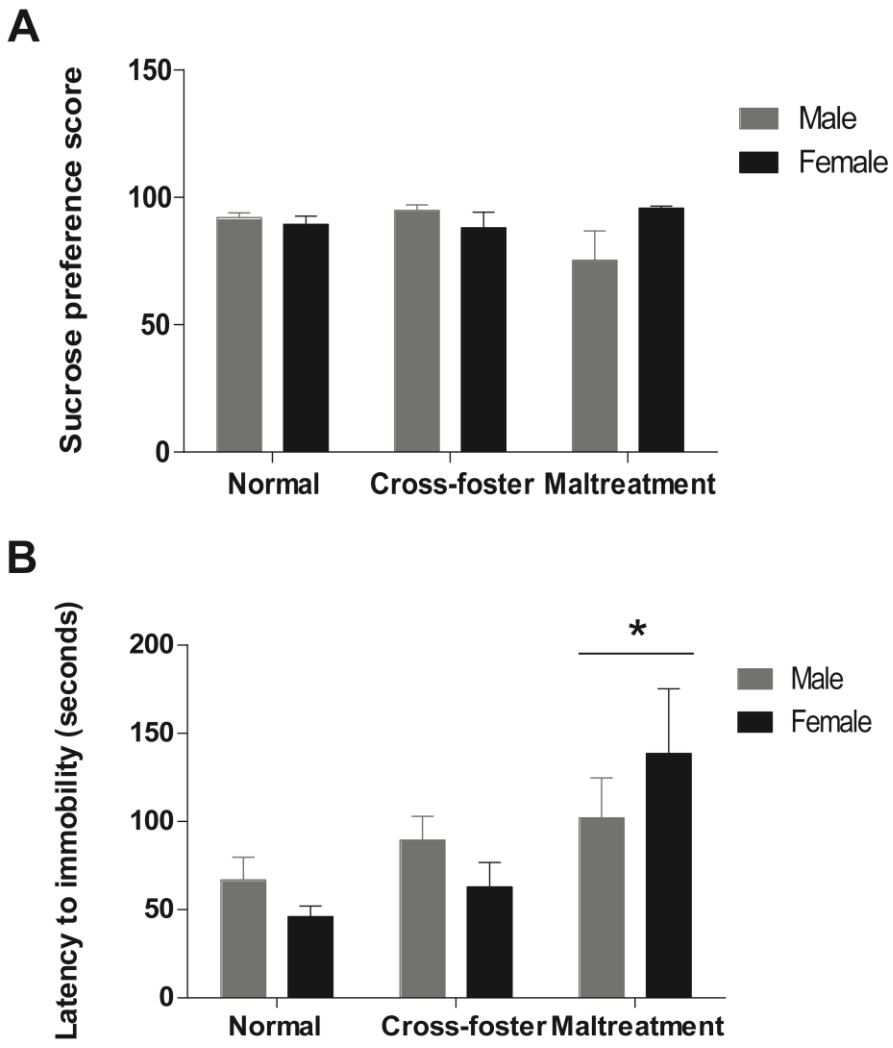


Figure 2.3 Sucrose preference scores (A) and latency to immobility during forced swim (B) in adult animals; n=9-12/group; *p<0.05 versus normal care controls; error bars represent SEM

In the realm of anxiety-like behavior, a two-way ANOVA (Figure 2.4A) revealed no main effect of infant condition ($F_{(2,63)}$=1.831, $p<0.05$) or sex ($F_{(2,63)}$=3.234, $p<0.05$), nor an interaction ($F_{(2,63)}$=1.587, $p<0.05$) on the latency to consume a food reward in the novelty suppressed feeding task. For marble-burying behavior (Figure 2.4B), two-way ANOVAs revealed no significant differences between infant conditions but did reveal a main effect of sex for buried ($F_{(1, 63)} = 9.992$, $p < 0.01$) and unburied ($F_{(1, 62)} = 13.15$, $p < .001$) marbles. However, these findings did not survive post-hoc analyses. Finally, a two-way ANOVA revealed a significant interaction between treatment and sex for time spent in the center of the open field (Figure 2.4C; $F(2,55)=4.791$, $p<0.05$). Post-hoc tests revealed that normal care males spent significantly more time in the center compared to normal care females ($p = 0.044$) and maltreated females ($p = 0.03$). There was also a significant interaction between caregiving condition and sex for number of entries into the center ($F(_{2,56})=3.435$, $p<0.05$), a finding that did not survive post-hoc analysis. There were no changes in locomotor activity between conditions or sexes (all p's>0.05; data not shown).

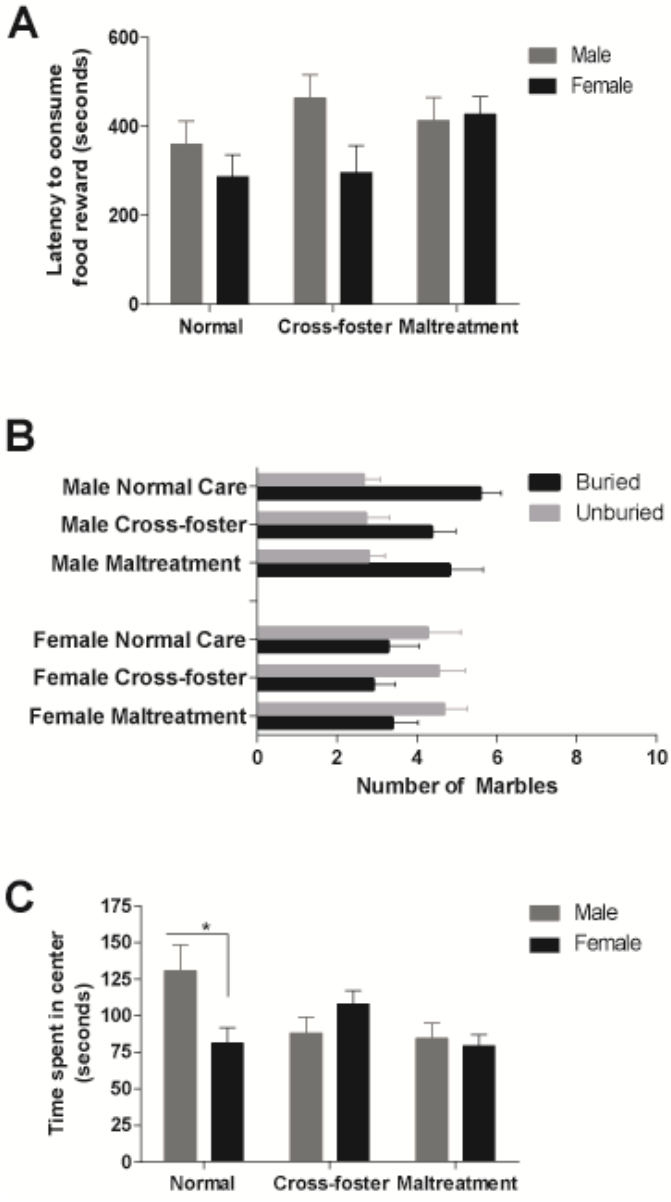


Figure 2.4 Latency to consume the food reward in a novelty suppressed feeding task is shown in adult male and female subjects (Graph a). Graph b represents the number of buried and unburied marbles in male and female adult subjects. Graph c illustrates the amount of time in seconds spent in the center of an open field during testing in male and female adult subjects. $n = 9–13$/group; $*p < 0.05$ males versus females in the normal care condition, error bars represent SEM

To assess fear-related learning and memory, adult rats were evaluated for fear conditioning, extinction, and retention (Figure 2.5). ANOVA conducted on fear conditioning training data yielded a main effect for blocked trial ($F_{(5,310)} = 333.168$, $p < .001$), indicating that fear conditioning was acquired in all subjects. There were no significant effects of sex or infant caregiver condition during fear conditioning (p's $> .05$), suggesting equivalent fear acquisition between both sexes and across all infant caregiver conditions. Analyses of fear extinction training revealed a sex X infant condition interaction ($F_{(2, 63)} = 5.724$, $p = .005$). To further examine this interaction, ANOVAs were performed on freezing levels for male and female subjects separately. These analyses revealed a main effect of condition for male subjects ($F_{(2, 31)} = 4.1$, $p = .026$). This main effect was mediated by enhanced levels of freezing in male subjects exposed to maltreatment in infancy relative to normal care controls ($p = .023$). There were no differences in freezing levels across infant conditions in females (p's $> .05$). These results indicate that males, but not females, exposed to maltreatment in infancy demonstrate deficits in the acquisition of fear extinction.

This deficit in fear extinction also persisted into fear extinction retention testing. ANOVA performed on fear extinction retention training data yielded a trial X sex X infant condition interaction ($F_{(10, 315)} = 1.985$, $p = .049$). Post-hoc analyses indicated that baseline freezing was significantly elevated in maltreated males versus their normal-care control counterparts ($p = .002$), while females neglected to show different levels of freezing during fear extinction retention testing across infant

caregiver conditions (p's > .05). This effect was male specific; there were no differences in freezing levels in females during the baseline of the fear extinction retention testing session (p's > .05). These results suggest that second order contextual fear conditioning could be occurring in males with a history of exposure to caregiver maltreatment, as they are acquiring fear to the fear extinction context (i.e. context B) even though the US was never presented in this context. This elevated fear to context B was not present prior to CS exposure in context B (i.e. there was no difference in freezing levels between animals from different infant caregiver conditions during the baseline session of fear extinction).

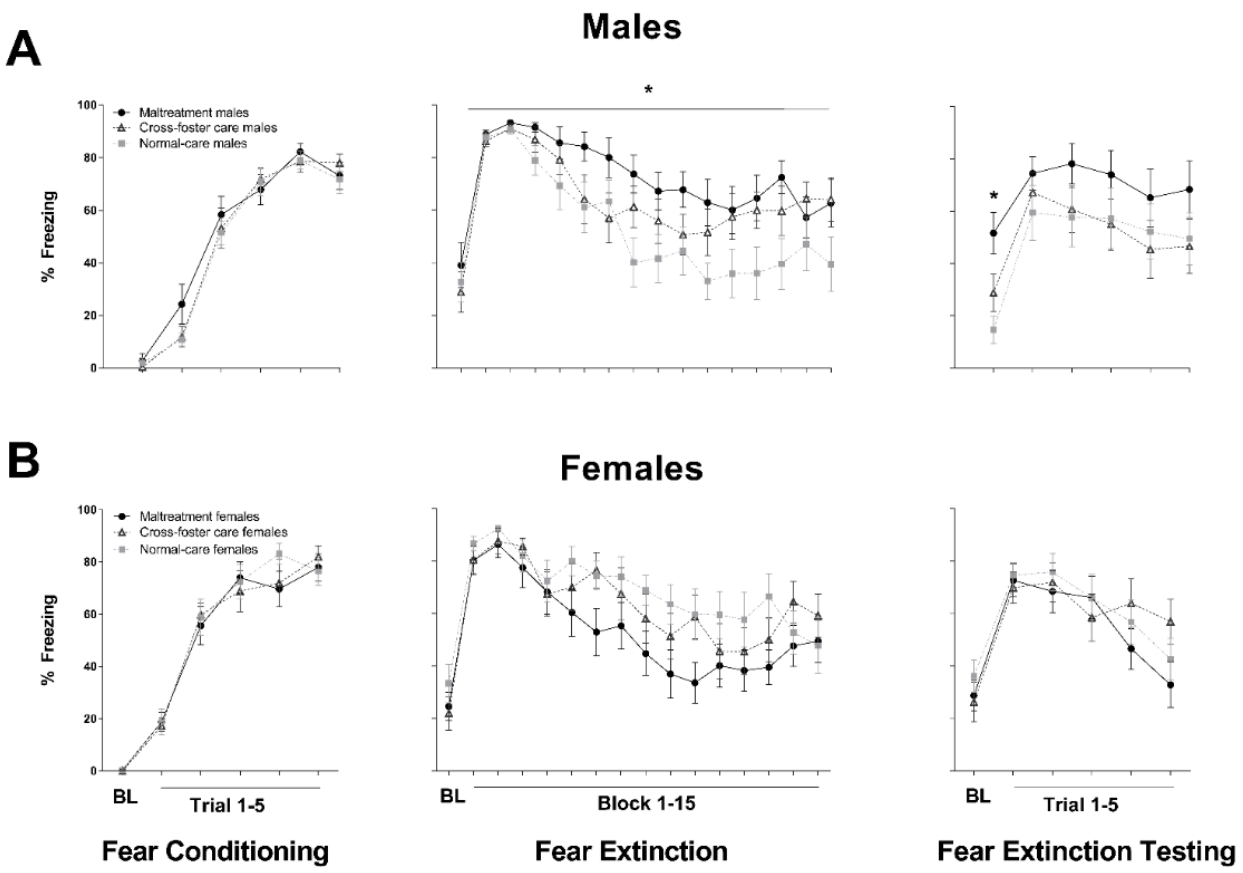


Figure 2.5. Freezing behavior during fear conditioning, extinction, and extinction retention testing in male (a) and female (b) adult rats; $n = 10–12$ group; $*p < 0.05$ versus normal-care controls; error bars represent SEM. BL: baseline.

Finally, NOR was used to evaluate recognition memory (Figure 2.6). During the sample phase (Day 1) both objects were equally explored by all rats (no main effect of infant condition ($p=0.7984$), sex ($p=.2326$), nor an interaction ($p=0.8397$); data not shown). An ANOVA conducted on data from the test phase (Day 2) revealed no main effect of infant condition ($p=0.7616$) or sex ($p=0.2994$), nor an interaction ($p=0.0970$). However, one-sample t-tests (typically used in this task to assess difference from chance, see section on statistical analysis) revealed that normal care ($t_{(10)}=4.511$, $p=0.0011$) and cross-foster care females ($t_{(10)}=4.082$, $p=0.0022$) could perform significantly above chance in the NOR, whereas maltreated females could not ($t_{(10)}=1.867$, $p=0.0915$). For males, the normal care ($t_{(9)}=2.434$, $p=0.0377$) and maltreatment ($t_{(9)}=2.288$, $p=0.0480$) conditions could perform the task above chance, but the cross-foster group could not ($t_{(8)}=0.3434$, $p=0.7402$). The inability of maltreated females to perform this task may be driven by variability (individual data points are provided in Figure 2.6). However, we have replicated this finding in additional cohorts in the lab associated with another on-going project (data unpublished).

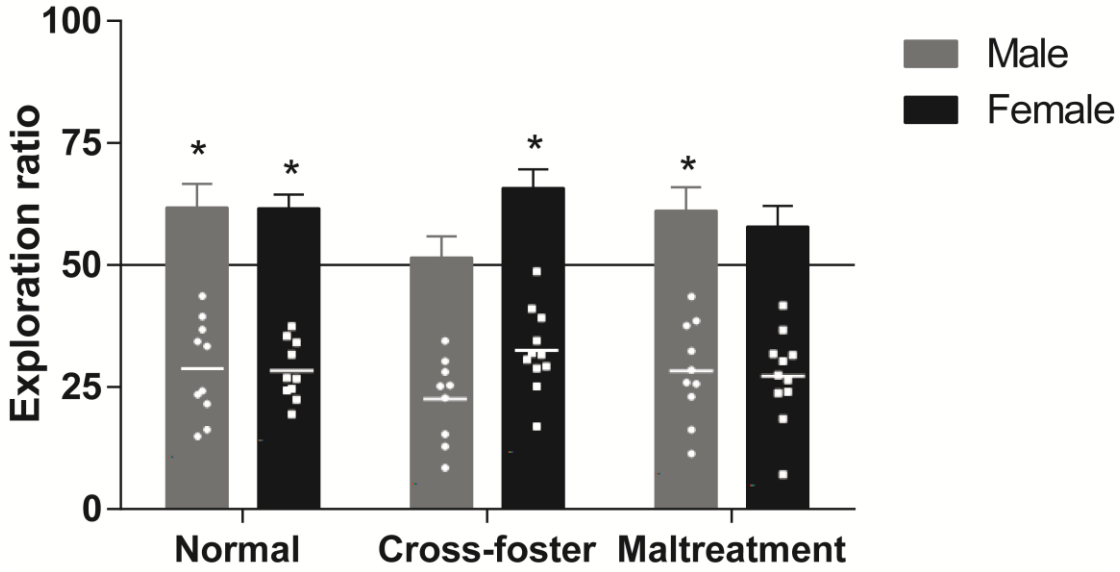


Figure 2.6 Exploration ratios in a novel object recognition task in male and female adult subjects along with individual subject points (Means: Normal care females = 61.80; cross-foster care females = 65.79; maltreated females = 57.87; normal care males = 61.79; cross-foster care males = 51.50; maltreated males = 61.12.); $n = 9–11$/group; $*p < 0.05$ versus 50% (chance); error bars represent SEM

2.4 Discussion

Here we characterized behavioral outcomes in the scarcity-adversity model of low nesting resources, a model of disrupted infant-caregiver interaction. Using a version of this model wherein caregiving manipulations take place outside the home cage, we found group- and sex-specific differences in behavior only at the adult time point. In our assessment of depressive-like behavior we found that maltreated animals exposed to an adverse caregiving environment exhibited a longer latency to immobility in the forced swim test. When assessing anxiety-like behaviors we found that exposure to nurturing or adverse care outside the home cage eliminated the sex-difference seen in normal-care controls in time spent in the center of an open-field arena. Assessment of recognition memory revealed that unlike their normal- and cross-foster care counterparts, maltreated females were unable to perform above chance in a novel-object recognition task.

No deficits were found in males when assessing anxiety-like behaviors with novelty-suppressed feeding and marble burying. However, when assessing performance on a fear conditioning and extinction paradigm, males displayed robust behavioral alterations not found in female subjects. Specifically, males exposed to maltreatment exhibited an extinction deficit such that they were significantly impaired at acquiring extinction when compared to their normal-care counterparts. This deficit persisted into extinction retention testing where they exhibited significantly higher levels of freezing at baseline than normal-care controls. Interestingly, in the novel-

object recognition task, cross-foster care males were unable to perform above chance, a deficit that was not found in their normal-care and maltreated counterparts. Though both tasks (novel-object recognition and fear extinction) assess learning and memory and both call on several of the same brain regions, male behavioral differences between groups on these tasks may be expected given the considerable element of fear in one task versus the other. Though difficult to surmise, the male cross-foster care deficit on the novel-object recognition task could be related to repeated removal from the home cage and subsequent exposure to a novel environment and dam, an effect that is somehow specific to this condition in which the novel environment is a nurturing one. The fact that this deficit is specific to the cross-foster condition in males will require further investigation to understand which factors are affecting performance in this task. Parameters of the cross-foster condition also appeared to have an effect on open-field performance. The sex difference seen in normal care controls was absent in both the cross-foster and maltreatment groups, suggesting that this effect is not specific to maltreatment.

Depending on one's interpretation of immobility in the forced swim test, the performance of our maltreated animals could be viewed in one of two ways. Immobility in this task has been interpreted as "behavioral despair" (Porsolt, Bertin, & Jalfre, 1978) in which case the behavior of our females could be considered adaptive given that their counterparts succumbed to behavioral despair more quickly. Another interpretation of this behavior is that it's a coping strategy allowing for energy conservation (West, 1990) in which case our data would suggest a decreased ability to

cope in animals exposed to maltreatment. Given that depressive- and anxiety-like behaviors are often comorbid (Grippo, Wu, Hassan, & Carter, 2008; Sartorius, Üstün, Lecrubier, & Wittchen, 1996) and that performance on other depressive (i.e. sucrose preference) and anxiety-like behavior (i.e. marble burying) assessments is on par with normal-care animals, the former explanation may be more likely. Further investigation will be necessary to determine which interpretation, if either, is appropriate. Previous work has also suggested deficits in the forced-swim task to be reflective of an inability to learn the immobility behavior (De Pablo, Parra, Segovia, & Guillamón, 1989). Considering that animals exposed to maltreatment exhibited behavioral deficits on tasks used to assess cognitive performance (i.e. novel-object recognition and fear learning/memory), the possibility that their forced-swim behavior is indicative of cognitive deficits also warrants further investigation.

Given that sucrose preference and forced swim tasks are often used in conjunction to assess depressive-like behavior, the opposing behavioral outcomes on these tasks is curious but not entirely surprising. The lack of difference between groups in sucrose preference makes sense in the context of some of the forced swim interpretations discussed above. For example, if forced swim differences are interpreted in the light of either adaptability or cognitive deficit, lack of difference in sucrose preference is less surprising. In addition, it is difficult to interpret the lack of differences on this task in our study given the widely variable results it produces based on factors such as rodent strain and experimental parameters such as timing of test (length of sucrose exposure as well as point of time after stressor), sucrose

concentration (Brenes Sáenz, Villagra, & Fornaguera Trías, 2006; Pothion, Bizot, Trovero, & Belzung, 2004; Van Dijken, Mos, van der Heyden, & Tilders, 1992). Indeed, the use of sucrose preference to adequately assess reward responsiveness after stress has been called into question (Forbes, Stewart, Matthews, & Reid, 1996). Overall, a much deeper investigation will be required to parse apart the behavioral outcomes reported here and what translational significance they may have.

Altered extinction behavior has been heavily implicated in anxiety disorders and conditions such as PTSD (Milad & Quirk, 2012), and these behaviors are often precipitated by early-life stress (Binder et al., 2008; Heim & Nemeroff, 2001; Heim et al., 2000; N. R. Nugent, Tyrka, Carpenter, & Price, 2011; Yehuda & Charney, 1993). The aberrant fear extinction behavior exhibited by males exposed to adverse caregiving are consistent with these data. The continuation of their aberrant freezing behavior into retention testing (i.e. higher levels of freezing at baseline during extinction retention testing) could be indicative of second-order contextual fear conditioning. Second-order conditioning entails the association of an innocuous stimulus with a conditioned stimulus (Pavlov, 1927; Rizley & Rescorla, 1972) and is associated with PTSD (Wessa & Flor, 2007). This is a poignant behavioral difference in our rats given the association between early life adversity and the development of PTSD following exposure to stressful stimuli later in life (McCranie, Hyer, Boudewyns, & Woods, 1992; Yehuda & Charney, 1993; Zaidi & Foy, 1994).

The lack of any behavioral differences in our adolescent animals is somewhat surprising, especially given that other early-stress paradigms disrupt behavior at this

time point (Bath, Manzano-Nieves, & Goodwill, 2016; Llorente et al., 2007; Marco et al., 2013; Molet et al., 2016), though the exposure time in these paradigms differs greatly from our own. Considering that stress exposure can lead to adaptive behavioral outcomes in some circumstances (Frankenhuis & de Weerth, 2013) (Bagot et al., 2009; D. L. Champagne et al., 2008; Oomen et al., 2010) it is possible that these brief, adverse exposures elicit compensatory mechanisms in adolescence, a stage where an incredible amount of restructuring takes place in the brain (Paus, 2005; Spear, 2000). For example, differential epigenetic marks (mechanisms heavily linked to behavioral outcomes following early stress) are seen in adolescence versus adulthood following early life stress (Blaze et al., 2013; Tiffany S Doherty et al., 2016; Roth et al., 2014) and reversal of epigenetic signatures after maternal separation stress is known to worsen adolescent behavior outcomes (A. Levine, Worrell, Zimnisky, & Schmauss, 2012).

The absence of differences at this age point may also have relevance to "sleeper effects", a phenomenon referred to in the clinical literature wherein children exposed to trauma exhibit no behavioral deficits in the short term but develop significant issues as time goes on (Elder & Rockwell, 1979; Freud & Burlingham, 1943; Wallerstein & Blakeslee, 1989). Data here support this as our rats exhibit behavioral abnormalities in adulthood on the same tests they successfully navigated in adolescence. Hormones may play a significant role in the latency of these behavioral deficits given that affective and stress-related behaviors are known to differ before and after puberty (Angold, Costello, & Worthman, 1998; Gomez, Manalo, & Dallman,

2004; Romeo, Lee, & McEwen, 2005). Along this line of reasoning, the sex differences found here support previous literature linking early adversity with differential biological and/or behavioral outcomes in females and males (Barr et al., 2004; Cirulli et al., 2009; Drury et al., 2012; Imanaka et al., 2008; McCormick, Smythe, Sharma, & Meaney, 1995; Pohl, Olmstead, Wynne-Edwards, Harkness, & Menard, 2007).

Characterization of behavioral phenotypes following early-life stress is critical even between similar models where experimental parameters are varied. For example, work from Regina Sullivan's lab using the scarcity-adversity model of low nesting resources within the home cage between postnatal days 8 and 12 has found depressive-like phenotypes in adolescent rats (Raineki et al., 2012). In contrast, we did not find any differences in depressive-like behaviors at this age with our experimental parameters (postnatal days 1 through 7, 30-minute exposures outside of the home cage). The Sullivan lab has also reported increases in the time spent immobile during the forced swim test in adult rats exposed to maltreatment in infancy (Raineki et al., 2015), findings that are also dissimilar from our own. These data help illustrate the importance of experimental variables like timing and severity of stress as factors in the risk for atypical outcomes and psychopathology.

In summary, the behavioral differences reported here help elucidate the utility of our model for investigating somewhat mild but repeated bouts of stress in the context of caregiving and its effect on the development of behavior. Such elucidation provides insight into the effects of differing stressors in development as we can

compare these results with those of other early stress models with varying parameters, including intensity and duration of stress. It is also useful in providing avenues for future investigation of the neurobiological mechanisms that underlie behavioral dysfunction evoked by early-life adversity.

Chapter 3

PREVENTION OF MALTREATMENT-INDUCED BDNF IX METHYLATION IN WHOLE PREFRONTAL CORTEX VIA HDAC INHIBITION

Reorganization of the brain's epigenetic landscape occurs alongside early adversity in both human and non-human animals. Whether this reorganization is simply incidental to or is a causal mechanism of the behavioral deficits that result from early adversity is important to understand. Using the scarcity-adversity model of low nesting resources in Long Evans rats, our lab has previously reported specific epigenetic and behavioral trajectories occurring in response to early disruption of the caregiving relationship. To further probe that relationship, the current work investigates the ability of the epigenome-modifying drug sodium butyrate to prevent maltreatment-induced methylation changes when administered alongside maltreatment. Following exposure to the scarcity-adversity model, during which drug was administered prior to each caregiving session, methylation of *Brain-derived Neurotrophic Factor* (*Bdnf*) IX DNA was examined in the Prefrontal Cortex (PFC) of male and female pups at postnatal day (PN) 8. As our previous work reports, increased methylation at this exon of *Bdnf* in the PFC is a stable epigenetic change across the lifespan that occurs in response to early maltreatment, thus giving us a suitable starting point to investigate pharmacological prevention of maltreatment-induced epigenetic

marks. Here we also examined off-target effects of sodium butyrate by assessing methylation in another region of *Bdnf* (exon IV) not affected in the infant brain as well as global levels of methylation in the brain region of interest. Results indicate that a 400 mg/kg (but not 300 mg/kg) dose of sodium butyrate is effective in preventing the maltreatment-induced rise in methylation at *Bdnf* exon IX in the PFC of male (but not female) infant pups. Administration of sodium butyrate did not affect the methylation status of *Bdnf* IV or overall levels of global methylation in the PFC, suggesting potential specificity of this drug. These data provide us with an avenue forward for investigating whether the relationship between adversity-induced epigenetic outcomes can be manipulated to improve behavioral outcomes.

3.1 Introduction

While it has long been established that caregiver-related adversity during development leads to lasting disruption of both physical (Danese et al., 2011; Danese et al., 2009; Dong et al., 2004; Felitti et al., 1998; Kiecolt-Glaser et al., 2011; Moloney, Stilling, Dinan, & Cryan, 2015) and mental health (Danese et al., 2009; Edwards, Holden, Felitti, & Anda, 2003; Felitti et al., 1998; Nanni, Uher, & Danese, 2012; Teicher & Samson, 2013), the molecular mechanisms underlying these disruptions remain a critical research focus. Effective treatment and intervention for individuals suffering from the effects of early adversity is dependent on our understanding of molecular events in the brain that contribute to poor physical and

mental health outcomes. Research within the last couple of decades has shed light on the brain's epigenetic response to adversity (Blaze & Roth, 2017; F. Champagne et al., 2006; Klengel et al., 2013; McGowan et al., 2009; Murgatroyd et al., 2009; Romens, McDonald, Svaren, & Pollak, 2015; Roth et al., 2009; Weaver et al., 2004; Weder et al., 2014), providing a rich mechanistic avenue of investigation in this area. The term epigenetics refers to a set of chemical alterations that change DNA output without changing the underlying sequence. The most well-known epigenetic mark, DNA methylation, occurs when a methyl group is added to DNA, often to the cytosine of a cytosine-guanine dinucleotide (L. D. Moore et al., 2013). Though this is known in some cases to lead to an increase in gene expression (Chahrour et al., 2008), it is more typically associated with significantly decreased expression (L. D. Moore et al., 2013).

The *Brain-derived Neurotrophic Factor* gene, or *Bdnf*, is intimately responsive to the caregiving environment (Bai et al., 2012; Daskalakis, De Kloet, Yehuda, Malaspina, & Kranz, 2015; de Lima et al., 2011; Gatt et al., 2009; Roceri, Hendriks, Racagni, Ellenbroek, & Riva, 2002; Suri et al., 2013) and is a major player in behavioral trajectories. It is critical to synapse function (Lu, Nagappan, & Lu, 2014), and its dysregulation is associated with negative behavioral outcomes related to both cognitive and mood disorders (Chen et al., 2006; Y.-K. Kim et al., 2007; Komulainen et al., 2008; Marais, Stein, & Daniels, 2009). Previous work from our lab demonstrates the highly dynamic epigenetic response of this gene to our paradigm of disrupted caregiving, and we have provided extensive evidence that it exhibits maltreatment-induced methylation changes that vary by age, sex, brain region, and exon examined

(Blaze & Roth, 2017; Blaze et al., 2013; Tiffany S Doherty et al., 2016; Roth et al., 2009; Roth et al., 2014), changes that may contribute to maltreatment-induced behavioral outcomes previously reported by our lab (T.S. Doherty, Blaze, Keller, & Roth, 2017; Keller, Doherty, & Roth, 2018; Roth et al., 2009). One of these changes, an increase in methylation at exon IX of *Bdnf* following early maltreatment, remains stable across the lifespan in the Prefrontal Cortex (PFC) (Roth et al., 2009). This stability provides us with an ideal starting point to investigate our ability to prevent epigenetic marks induced by our maltreatment paradigm, with the eventual goal of preventing abnormal behavioral outcomes. While it is known that treatments affecting the epigenome can attenuate behavioral abnormalities induced by early adversity (Roth et al., 2009; Valvassori et al., 2014; Weaver et al., 2004; Weaver, Meaney, & Szyf, 2006), most studies have focused on adulthood, long after the adverse event and subsequent epigenetic reorganization have occurred. The question posed by the current study focuses on the possibility of preventing these marks from occurring in the first place, an initial step in a path aimed at understanding if we can prevent them (i.e. across the lifespan) and what that means for behavioral deficits that manifest long after the initial insult.

A variety of drugs are known to alter epigenetic states via actions on the enzymes that catalyze epigenetic changes. While many of these drugs were first used in cancer therapy, they may be effective in treating various psychopathologies (Grayson, Kundakovic, & Sharma, 2010; Shepard et al., 2018; Szyf, 2009; Varela et al., 2015) and physical health problems following early adversity (Moloney et al., 2015).

Sodium butyrate is one of these drugs. It is known as a histone deacetylase inhibitor (HDACi) due to its ability to prevent the actions of histone deacetylase enzymes, or HDACs (Boffa, Vidali, Mann, & Allfrey, 1978). These enzymes remove acetyl groups from histones, the proteins around which DNA wraps. This action alters the charge of the histone protein and results in a tighter coupling between the histone and its associated DNA (i.e. a tighter chromatin complex), resulting in a less permissive transcriptional state. Thus, administration of a HDACi such as sodium butyrate should facilitate a more permissive transcriptional state given its ability to suppress the enzymes responsible for a tighter, less accessible chromatin structure. In terms of methylation, levels may be indirectly altered by HDACi administration due to crosstalk between levels of acetylation and methylation (Cervoni & Szyf, 2001). In addition, sodium butyrate is known to affect methylation levels via its downstream effects on DNMT1, one of the enzymes that catalyzes DNA methylation (S. Sarkar et al., 2011). Lastly, though one would expect that peripheral administration of a HDACi would have global implications within the epigenome, data suggest that only a portion of genes are subject to their effects (Weaver et al., 2006; W. S. Xu, Parmigiani, & Marks, 2007). Thus, though the mechanisms are poorly understood, these drugs exhibit a level of specificity in their actions.

Given previously mentioned data from our lab demonstrating increased *Bdnf* methylation following adversity, and taking into account that HDAC activity is higher in adversity-exposed brains (Albuquerque-Filho et al., 2017) and *Bdnf* is a gene selectively altered by sodium butyrate (Barichello et al., 2015; Shepard et al., 2018;

Varela et al., 2015; X. Wu et al., 2008), we chose to employ this HDAC inhibitor to investigate the feasibility of preventing aberrant epigenetic marking of the *Bdnf* gene in the adversity-exposed brain. Thus, the current study examines *Bdnf* exon IX methylation in the male and female PFC of developing animals following exposure to nurturing or adverse care during the first week of life, and the ability of the HDACi sodium butyrate to prevent the previously reported, adversity-induced increase in methylation at this locus.

3.2 Methods

All procedures were conducted by the standards and with the approval of the University of Delaware Animal Care and Use committee.

3.2.1 Subjects and Caregiving Manipulations

Long Evans rat mothers and pups (24 litters total, 9-12 pups per litter) were housed in 18”x9’x8’ polypropylene cages with ample bedding and *ad libitum* access to food and water in a light- and temperature-controlled room (12-hour light/dark cycle with lights on at 6:00 am). All experimental procedures were done during the light cycle. Dams were bred in the lab and allowed to have one litter before they were used in experimental paradigm, alleviating potential confounds associated with being a first-time mother. Manipulations began on PN1, thus leaving dams and pups undisturbed on the day of birth (PN0). On PN1 litters were culled to 5-6 males and 5-6 females.

Pups were then randomly assigned to one of three caregiving conditions, with one male and one female per caregiving condition randomly assigned to vehicle or drug, resulting in one subject per litter being assigned to each experimental group. All procedures were approved by the University of Delaware Animal Care and Use Committee prior to beginning the experiment.

Infant rats were exposed to their respective caregiver condition for 30 minutes per day for the first seven days of life (PN1-PN7). Pups were randomly assigned to either the maltreatment, cross-foster care, or normal-care condition. In the maltreatment condition, pups were exposed to a lactating dam who had been placed in a novel environment with limited nesting material and no time to habituate to her environment, conditions which elicit stress-induced, erratic and aversive caregiving behaviors (Ivy et al., 2008; Perry et al., 2018; Raineki, Moriceau, & Sullivan, 2010; Roth et al., 2009). In the cross-foster care condition, pups were similarly exposed to a lactating dam who had been placed in a novel environment, but provided with ample nesting material and plenty of time to habituate (approximately 1 hour). In the normal-care condition, pups were kept in the home cage to be cared for by their biological mother, removed only for weighing, marking, and saline/drug administration.

All caregiving sessions were recorded on video and a randomly chosen subset was later scored by two trained observers who scored nurturing (hovering and nursing, licking and grooming) and aversive (stepping on, dropping, dragging, actively avoiding, or roughly handling) caregiving behaviors. These behaviors were marked as having occurred or not during 5-minute time bins over the course of each 30-minute

session and the scores of the two observers were then averaged together. The resulting scores for each caregiving behavior were then averaged across the seven sessions for statistical analysis. Pup ultrasonic vocalizations (40kHz) were also recorded during experimental sessions and scored/analyzed similarly, but in 1-minute time bins across each 30-minute session.

3.2.2 Drug Preparation and Administration

Sodium butyrate (first cohort given 300 mg/kg, second cohort in subsequent experiment given 400 mg/kg) was dissolved in a 5% sucrose solution and delivered orally to pups with a pipette (vehicle animals were given only the 5% sucrose solution), based on their current weight, prior to each caregiving session.

3.2.3 *Bdnf* Methylation

Prefrontal cortical tissue was harvested and homogenized for nucleic acid extraction according to the manufacturer's instructions (Qiagen AllPrep DNA/RNA kit) and then bisulfite modified (Qiagen Inc.). *Bdnf* methylation levels were determined using methylation-specific real-time PCR (MSP; Bio-Rad CFX96 system). Bisulfite-modified DNA was amplified using methylated primer sets associated with exons IV and IX (Roth et al., 2009). The relative fold change of cross-fostered and maltreated animals versus normal-care animals was obtained using the comparative C_t

method (Livak & Schmittgen, 2001). Tubulin was used as a reference gene and product specificity was confirmed via melt curve analysis and gel electrophoresis.

3.2.4 Global Methylation

The same PFC-derived DNA used for locus-specific assays was used to assess global methylation. MethylFlash™ Methylated DNA Quantification Kits were used to quantify levels of genome-wide methylation (5-mC) according to the manufacturer's instructions (Epigentek, Brooklyn, NY), with the only deviation being the added step of mechanically shaking the plate (using a plate reader with this function) immediately after each step where a new component has been added. Using this kit, capture and detection antibodies are employed to detect methylated DNA which is then colorimetrically quantified (via measurement of light absorption) against a standard curve formed with control DNA provided with the kit. Absorbance was measured using the Infinite ® F50 microplate reader (Tecan, Männedorf, Switzerland) with the amount of 5-mC being proportional to the intensity of the optical density. Samples were run in vertical duplicates at a strict concentration of 100 ng/well with total volume added per well not ranging outside of 2-5 ul.

3.2.5 Statistical Analyses

Behavioral data were analyzed using one- and two-way ANOVA (two-way factors: caregiving condition and caregiver behavior). Group differences in gene-

specific methylation and global 5mC levels were analyzed using three-way ANOVAs (drug, sex, caregiving condition) and one-sample t-tests. Post-hoc tests (Tukey's and Fisher's LSD) were performed where appropriate. For all analyses, differences were considered to be statistically significant for $p < 0.05$.

3.3 Results

3.3.1 Caregiving Manipulations

Adverse and nurturing behaviors collapsed across the seven days for each condition are illustrated in Figure 3.1, with specific behaviors illustrated in the pie charts. A two-way ANOVA of nurturing vs adverse behaviors revealed a main effect of behavior type ($F_{1,18} = 38.84$, $p<0.001$) and a behavior type x infant condition interaction ($F_{2,18} = 28.05$, $p<0.001$). Post-hoc analyses revealed that maltreated animals experienced significantly higher levels of adverse caregiving and significantly lower levels of nurturing caregiving than both cross-foster ($p=0.0012$) and normal-care ($p=0.0004$) controls. One-way ANOVAs revealed a significant effect of infant condition on the level of hovering/nursing ($F_{2,9} = 8.061$, $p<0.01$), actively avoiding pups ($F_{2,9} = 7.628$, $p<0.05$), and roughly handling pups ($F_{2,9} = 8.821$, $p<.01$). No significant differences in licking/grooming ($p=0.6231$), stepping ($p=0.1266$), dropping ($p=0.0600$), or dragging ($p=0.5621$) were found between groups. Tukey's post-hoc tests revealed that when compared to normal- ($p=0.0183$) and cross-foster care ($p=0.0163$) controls, maltreated pups experienced significantly lower levels of

hovering/nursing. Compared to normal- ($p=0.0298$) and cross-foster care ($p=0.0079$) controls, maltreated pups also experienced higher levels of rough handling during manipulations, and significantly more avoidance behavior from the dam than pups in the normal ($p=0.0146$) and cross-foster ($p=0.0294$) care conditions.

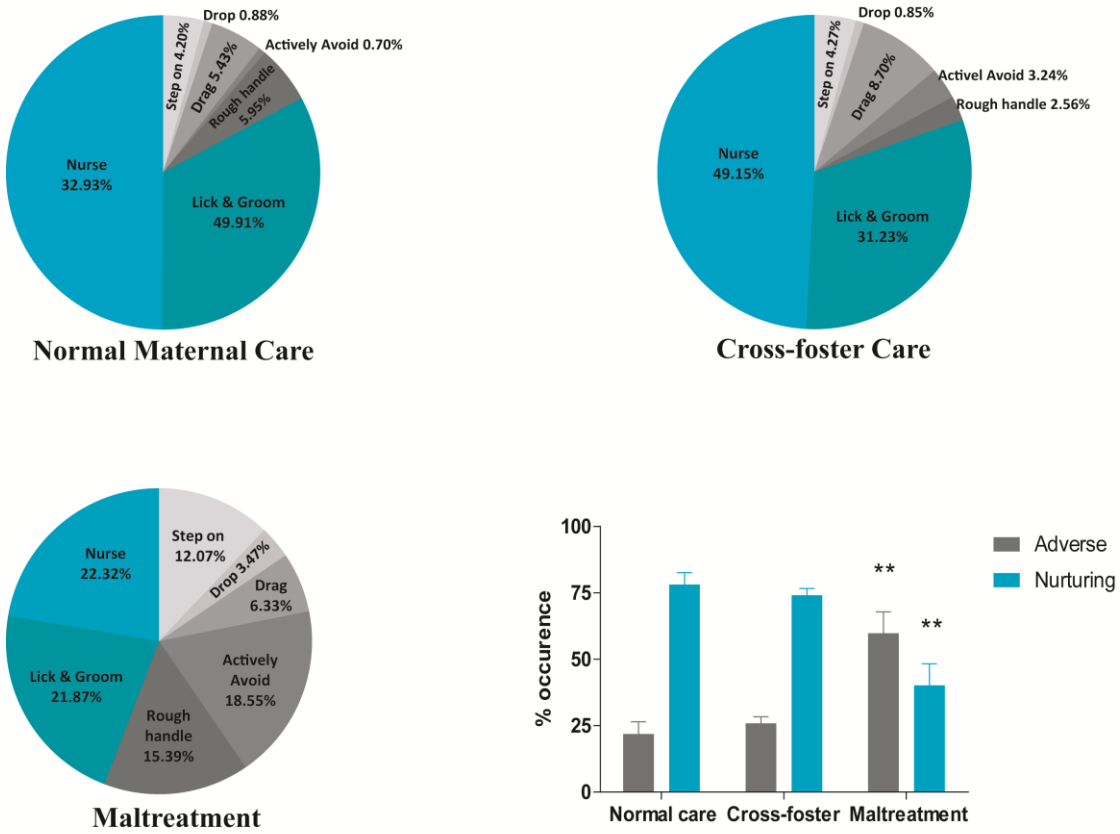


Figure 3.1 Breakdown of nurturing and adverse maternal behavior in each infant condition. Bar graph depicts nurturing versus adverse maternal behaviors collapsed across all seven days of paradigm for each condition. N=4 litters/condition. **p<0.001 vs. normal- and cross-foster care controls. Error bars represent SEM.

Analysis of pup ultrasonic vocalizations (Figure 3.2) also revealed a significant difference between infant conditions ($F_{2,9}$ =5.005, p=0.0346), with maltreated pups emitting significantly more vocalizations than both normal- (p=0.0248) and cross-foster care (p=0.0211) controls.

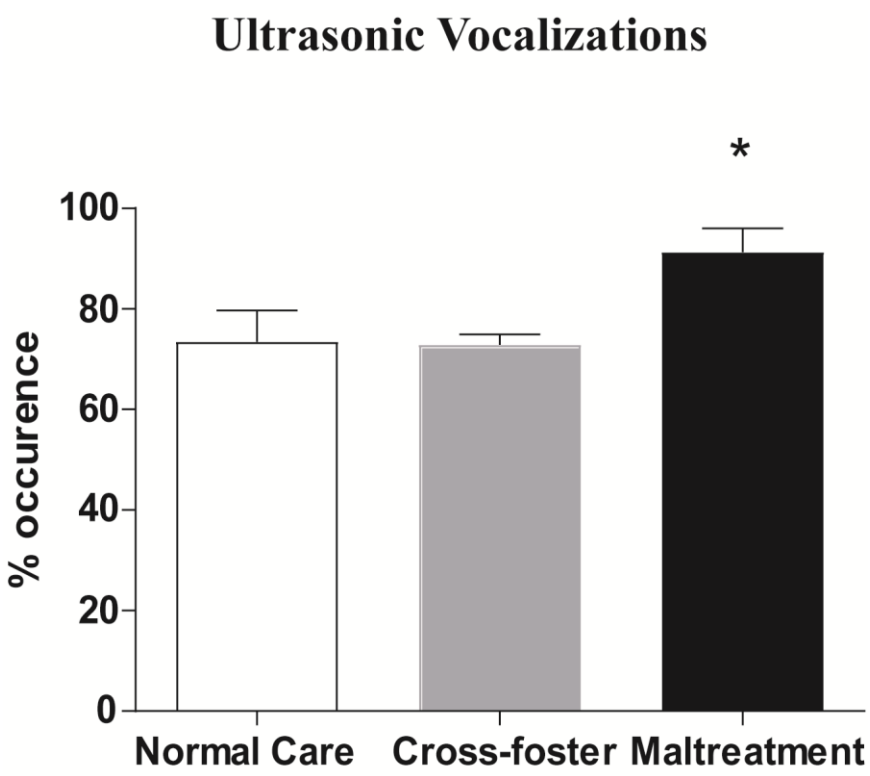


Figure 3.2 Ultrasonic vocalizations emitted by pups during caregiving sessions. N=4 litters/condition. *=$p<0.05$. Error bars represent SEM.

3.3.2 *Bdnf* Methylation

As illustrated in Figure 3.3A for the 300 mg/kg experiment, and as previously reported (Roth et al., 2009) vehicle-treated males (t_9=3.572, p=0.0060) and females (t_9=3.561, p=0.0061) in the maltreatment group exhibit significantly higher levels of methylation when compared directly to vehicle-treated, normal-care control counterparts (represented by dashed line at 1). This analysis in the 300 mg/kg drug groups revealed the same effect: drug-treated males (t_{10}=3.18, p=0.0098) and females (t_{11}=3.659, p=0.0038) in the maltreatment group exhibit significantly higher levels of methylation when compared directly to vehicle-treated, normal-care control counterparts, suggesting that sodium butyrate at this dose is ineffective at preventing maltreatment-induced exon IX methylation rises. Results reveal a main effect of infant condition ($F_{1,72}$ =16.42, p<0.001) and sex ($F_{1,72}$ =8.716, p<0.01), with post-hoc analyses indicating lower levels of methylation in male versus female subjects (p<0.05) and higher levels of methylation in maltreated versus control subjects (p<0.05). Results reveal no main effect of drug ($F_{1,72}$ =0.001676, p=0.9675) and no interaction of infant condition and drug ($F_{1,72}$ =0.325, p=0.5704), drug treatment and sex ($F_{1,72}$ =0.05909, p=0.8086), infant condition and sex ($F_{1,72}$ =1.866, p=0.1761), or an infant condition x drug x sex interaction ($F_{1,72}$ =1.501, p=0.2245).

As illustrated in Figure 3.3B for the 400 mg/kg experiment, and as previously reported (Roth et al., 2009), vehicle-treated males (t_9=2.615, p=0.0280) and females (t_9=3.882, p=0.0037) in the maltreatment group exhibit significantly higher levels of

methylation when compared directly to vehicle-treated, normal-care control counterparts. This analysis in the 400 mg/kg drug groups revealed that drug-treated females also exhibit this increase (t_{10}=3.566, p=0.0051), but males do not (t_{10}=1.078, p=0.3065), suggesting that this sodium butyrate at this dose is effective at preventing maltreatment-induced exon IX methylation in males but not females. Results reveal no main effect of drug ($F_{1,72}$ =2.469, p=0.1205) and no interaction of infant condition and drug ($F_{1,72}$ =0.03504, p=0.8520), drug treatment and sex ($F_{1,72}$ =0.1072, p=0.7443), infant condition and sex ($F_{1,72}$ =0.8916, p=0.3482), or an infant condition x drug x sex interaction ($F_{1,72}$ =1.363, p=0.2469). Results did reveal a main effect of infant condition ($F_{1,72}$ =11.06, p<0.01) and sex ($F_{1,72}$ =15.81, p<0.001), with post-hoc analyses indicating lower levels of methylation in male versus female subjects (p<0.05) and higher levels of methylation in maltreated versus control subjects (p<0.05).

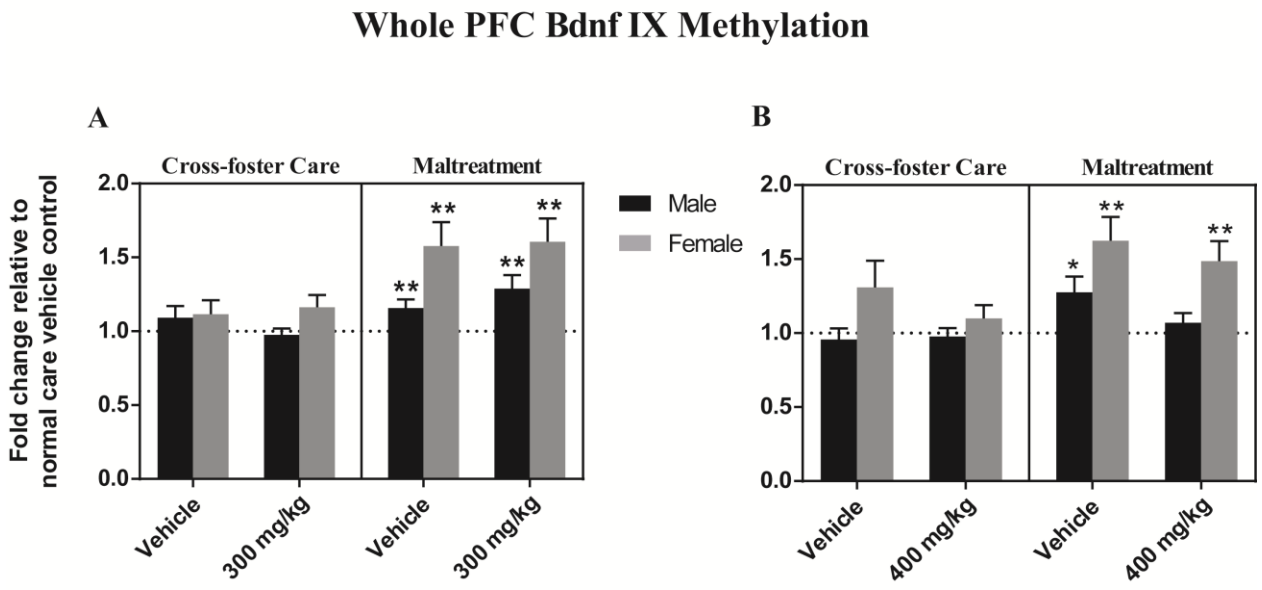


Figure 3.3 *Bdnf* IX methylation fold change in whole PFC in 300 mg/kg (A) and 400 mg/kg (B) cross-foster and maltreatment groups when compared to normal care vehicle control. N=10-11 p/group. *=p<0.05, **=p<0.01. Error bars represent SEM.

To determine if sodium butyrate administration was affecting methylation within other regions of the *Bdnf* gene in the 400 mg/kg cohort, we examined exon IV (Figure 3.4), an exon at which methylation levels in the PFC are not affected by maltreatment at this age (Roth et al., 2009). As reported in previous studies, no significant differences were found at this exon when comparing maltreated animals directly to vehicle-treated, normal-care control counterparts (all p's>0.05). Likewise, no differences were found in vehicle- or drug-treated groups in the current study (all p's>0.05). A three way ANOVA revealed no main effect of sex ($F_{1,72}$ =0.0964, p=0.7571) or infant condition ($F_{1,72}$ =0.244, p=0.6228), and no interactions (all p's>0.05). Although a main effect of drug was revealed ($F_{1,72}$ =4.089, p=0.0469), this did not survive post-hoc analysis.

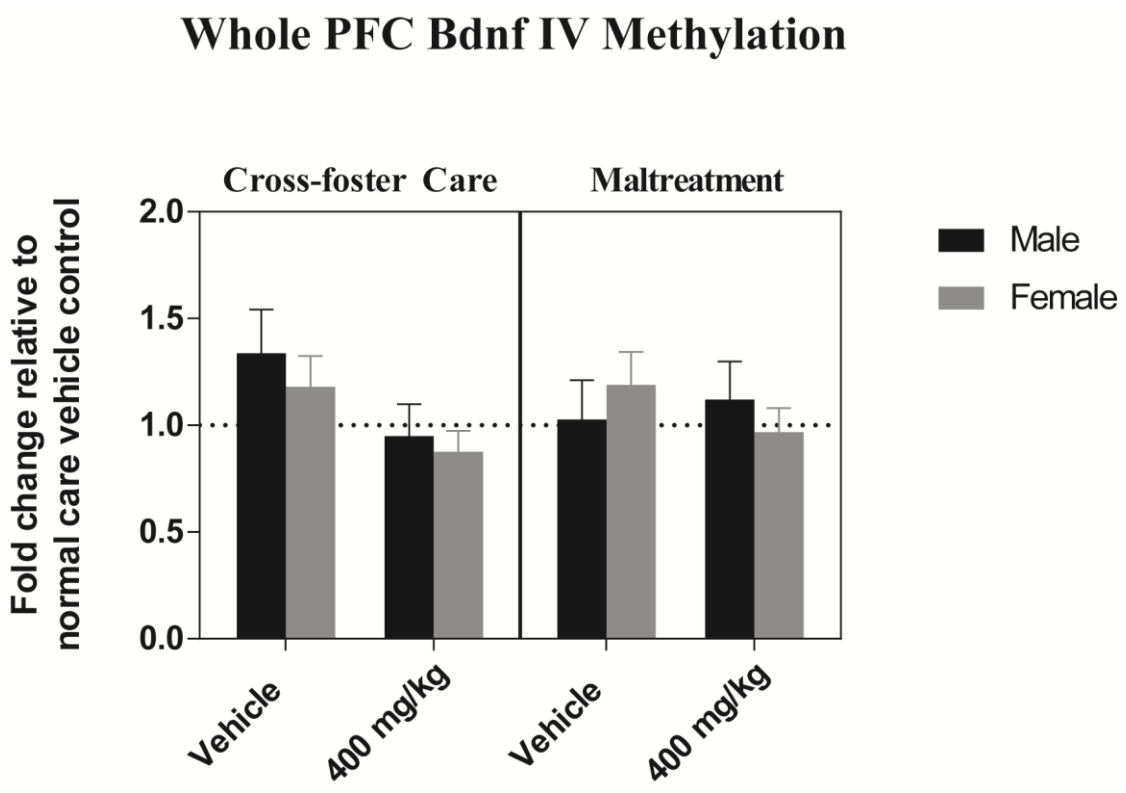


Figure 3.4 *Bdnf* IV methylation fold change in whole PFC in 400 mg/kg cross-foster and maltreatment groups when compared to normal care vehicle control. N=10-11 p-group. Error bars represent SEM.

3.3.3 Global Methylation

Percent global methylation levels in the 300 mg/kg sodium butyrate experiment (Figure 3.5A) were analyzed with a three way ANOVA. Results reveal no main effect of drug ($F_{1,96}$ =2.196, p=0.1416) and no interaction of infant condition and drug ($F_{2,96}$ =0.4385, p=0.6463), infant condition and sex ($F_{2,96}$ =2.989, p=0.0551), drug treatment and sex ($F_{2,96}$ =0.1202, p=0.7296), or an infant condition x drug x sex interaction ($F_{2,96}$=0.6383, p=0.5304). Though results did reveal a main effect of sex ($F_{1,96}$ =6.949, p<0.01) and infant condition ($F_{2,96}$ =5.584, p<0.01), these did not survive post-hoc analysis.

Likewise, percent global methylation levels in the 400 mg/kg sodium butyrate experiment (Figure 3.5B) were analyzed with a 2x2x3 ANOVA. Results reveal no main effect of drug ($F_{1,108}$ =0.3854, p=0.5360), sex ($F_{1,108}$ =1.498, p=0.2236), or infant condition ($F_{2,108}$ =0.4341, p=0.6490). Likewise, results indicated no interaction of infant condition and drug ($F_{2,108}$ =1.143, p=0.3228), infant condition and sex ($F_{2,108}$ =1.162, p=0.3166), drug treatment and sex ($F_{1,108}$ =1.709, p=0.1939), nor an infant condition x drug x sex interaction ($F_{2,108}$ =0.2714, p=0.7628).

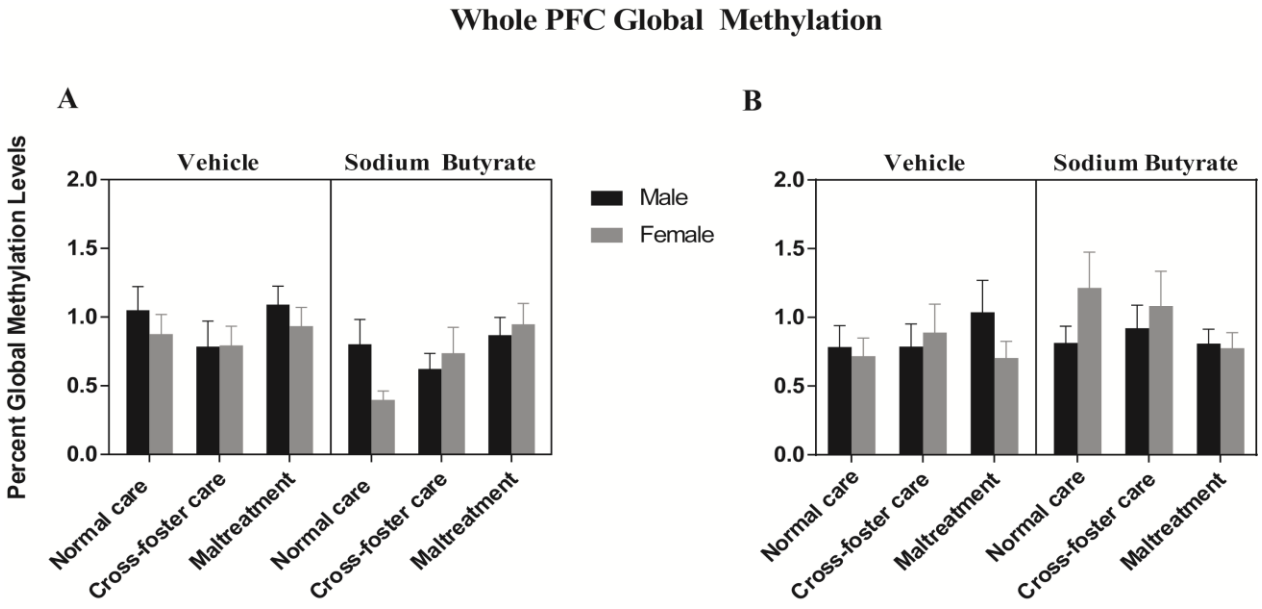


Figure 3.5 Percent global methylation change in whole PFC in 300 mg/kg (A) and 400 mg/kg (B) cross-foster and maltreatment groups. N=10-11 p/group. Error bars represent SEM.

3.4 Discussion

Taken together, the data presented here reinforce our previous reports of adversity-induced epigenetic marking of the *Bdnf* gene in response to brief, repeated bouts of maltreatment (Roth et al., 2009), and demonstrate the feasibility of preventing these marks. Specifically, daily administration of a 400 mg/kg (but not a 300 mg/kg) dose of the HDACi sodium butyrate, concurrent with exposure to our maltreatment paradigm, prevented the previously reported increase in methylation at *Bdnf* exon IX in male (but not female) PFC. Methylation status at exon IX was unaffected by sodium butyrate in normal- and cross-foster care animals. Additionally, examination of methylation levels at another *Bdnf* exon (exon IV) as well as global levels of methylation in the PFC revealed no changes.

The current study adds to a small but important area of literature investigating prevention of maltreatment-induced epigenetic marks before they can manifest in the brain and perpetuate into adulthood. As previously discussed, the therapeutic potential of drugs that can act on the epigenome has been assessed in the context of early adversity (Roth et al., 2009; Valvassori et al., 2014; Weaver et al., 2004; Weaver et al., 2006), but has focused on attenuation of already-manifested deficits in adulthood. We believe that investigating the preventative potential of such drugs is equally promising, both in terms of intervention and in terms of understanding the intricate relationship between the epigenome and behavior. Indeed, previous work demonstrates both epigenetic and behavioral rescue by HDAC inhibition when administered in infancy

alongside caregiver-related adversity (Burenkova, Aleksandrova, & Zarayskaya, 2019; Kao et al., 2012; A. Sarkar et al., 2014).

We have previously reported sex-specific behavioral effects of maltreatment on various realms of behavior (T.S. Doherty et al., 2017), deficits that we hypothesize are related to altered methylation of *Bdnf*. Given that *Bdnf* is among the genes selectively affected by sodium butyrate (Barichello et al., 2015; Varela et al., 2015; X. Wu et al., 2008), and that use of such drugs is effective in many cases at rescuing these types of behavioral deficits in adulthood (Govindarajan, Agis-Balboa, Walter, Sananbenesi, & Fischer, 2011; Reolon et al., 2011; Schroeder et al., 2007; Valvassori et al., 2014), the current results suggest great potential in preventing abnormal behavioral development in our male maltreated rodents.

At the current doses we were unable to prevent the increase in methylation at exon IX brought on by maltreatment in the female PFC. Though we are currently unsure of the mechanism underlying the sex difference in our findings, we hypothesize that it is related to a combination of a couple different factors. First, there are sex differences in basal levels of epigenetic modifications (Kurian, Olesen, & Auger, 2010; B. M. Nugent et al., 2015; Tsai, Grant, & Rissman, 2009) and enzymes (Blaze & Roth, 2013; Kigar, Chang, Hayne, Karls, & Auger, 2016; Kolodkin & Auger, 2011; Kurian, Forbes-Lorman, & Auger, 2007; J. Xu, Deng, & Disteche, 2008; J. Xu, Deng, Watkins, & Disteche, 2008), which may affect the female epigenetic response to both maltreatment and epigenome-modifying drugs. Second, recent data from our lab show that females experience a higher proportion of adverse care in our maltreatment

paradigm than do male pups (Keller, Nowak, & Roth, 2019). Given that male pups are subject to increased nurturing (licking) behavior in the home cage (C. L. Moore & Morelli, 1979; Richmond & Sachs, 1984), females subject to our brief exposures of maltreatment are overall experiencing more adverse and less nurturing behaviors than male pups subject to the same exposures. This may translate to a more robust epigenetic response in females that requires a more robust intervention. Current work in the lab is assessing this possibility with other epigenetic agents at varying doses.

Assessment of methylation status at another region of *Bdnf* (exon IV) as well as global levels of methylation in the maltreated PFC revealed no drug-induced changes in methylation, suggesting that sodium butyrate's effects were specific to maltreatment-induced epigenetic abnormalities. Alongside our data revealing no effect of drug on control (cross-fostered or normal-care) animals in the current measures, these results paint a broad picture of experimental and therapeutic promise. However, given that sodium butyrate and other HDACi drugs can act on multiple processes (Gagliano, Delgado-Morales, Sanz-Garcia, & Armario, 2014; Kruh, 1981), our lab is assessing other off-target effects of this drug in ongoing experiments.

Taken together, these data hint at the utility of epigenetic drug intervention in developmental adversity, and highlight the importance of studying sex differences in such cases. Current work in the lab is assessing epigenetic and behavioral outcomes in adulthood following drug intervention in infancy to understand if the current effects persist across the lifespan, and what implications that may have for maltreatment-induced behavioral abnormalities. Overall, the current study is an initial step forward

in our attempt to understand the relationship between the epigenome and behavior in the context of a disrupted caregiving environment.

Chapter 4

SUMMARY AND IMPLICATIONS OF FINDINGS

The data reported in this dissertation reinforce and extend evidence for the hypothesis that exposure to our model of early adversity catalyzes altered behavioral trajectories in offspring long after the initial insult. Specifically, experiments conducted in Chapter 2 demonstrate sexually dimorphic behavioral deficits in adult (but not adolescent) maltreated animals. The current data also reinforce a growing body of literature implicating epigenetic mechanisms in this relationship and the therapeutic potential of pharmacological agents that act on the epigenome. Specifically, experiments conducted in Chapter 3 replicate the previously reported *Bdnf* IX methylation increase in the PFC of maltreated animals. In addition, experiments in Chapter 3 demonstrate the ability of the HDACi sodium butyrate (at 400 but not 300 mg/kg) to prevent this increase when administered alongside maltreatment. Interestingly, this dose was only able to prevent methylation at this locus in the male brain.

Though several studies have reported altered behavior in adolescent animals following early stress exposure, we did not find any maltreatment-induced behavioral deficits in adolescent animals on the tasks we examined. Though it is difficult to speculate why our paradigm of adversity did not elicit behavioral alterations here, it is possible that we simply did not capture the effects of adversity in the handful of tasks we chose for testing. It is also possible that our maltreatment paradigm elicits compensatory mechanisms in the adolescent but not adult brain. As discussed in

Chapter 2, children exposed to trauma sometimes exhibit serious behavioral pathology in the long- but not short-term, a phenomenon referred to as "sleeper effects" (Elder & Rockwell, 1979; Wallerstein & Blakeslee, 1989). Further, it could be the case that the maltreatment-induced methylation increase that we report at exon IX is protective in adolescence. Indeed, reversal of epigenetic marks caused by maternal separation actually worsens behavioral outcomes in adolescent subjects (A. Levine et al., 2012). Further investigation of these points will be critical given that our overarching hypothesis is that altered *Bdnf* methylation contributes to maltreatment-induced behavioral deficits. Further understanding of these points will also be critical to potential epigenetic therapies; drug intervention parameters may need to be altered based on age in order to achieve effective outcomes.

The experiments conducted in Chapter 3 are a broad-stroke approach to understanding our ability to manipulate the epigenome, particularly in terms of its response to adverse events. This is a necessary step in our quest to better understand the relationship between adversity, epigenetic alterations, and later behavioral outcomes. The data reported in Chapter 3 provide us with a starting point to investigate the behavioral implications of manipulating the epigenetic response to adversity. Our lab may now combine the information provided by Chapters 2 and 3 of this dissertation to directly assess the behavioral effects of preventing adversity-induced epigenetic marks.

Though the epigenome responds to adversity across numerous brain regions, this investigation was limited to the PFC. We confined our current work to this region because while *Bdnf* IX methylation varies by age and sex in response to our paradigm in regions such as the amygdala and hippocampus, methylation levels at this exon

exhibit consistent adversity-induced increases in the PFC regardless of age and sex. Thus, examination of the PFC provides us with a stable point of investigation across experiments, lessening the number of factors to take into account for establishing proof-of-principle (i.e. that we could use this particular epigenetic agent to prevent adversity-induced methylation). Thus, the current results do not shed light on the effects of HDACi in the rest of the adversity-exposed brain, though ongoing work in our lab is addressing this. That being said, the significance of PFC rescue should not be understated. This brain region is directly affected by early-life adversity (Hanson et al., 2012; Roth et al., 2009; Spinelli et al., 2009; Uchida et al., 2010) and its dysfunction is directly implicated in performance deficits on the tasks examined in chapter 2 (Banasr & Duman, 2008; Milad & Quirk, 2002; Santini, Ge, Ren, de Ortiz, & Quirk, 2004; Watson et al., 2011). Altered *Bdnf* levels in this brain region are also implicated in performance of these tasks (Bredy et al., 2007; Camer et al., 2015; Zhou et al., 2014).

Our work with sodium butyrate will need to continue to establish an effective dose in females. It is not surprising that males and females differ in their response to an epigenetic agent. As discussed in Chapter 3, the epigenetic landscape at baseline, along with basal levels of epigenetic enzymes, vary between males and females (Blaze & Roth, 2013; Kigar et al., 2016; Kolodkin & Auger, 2011; Kurian et al., 2007; Kurian et al., 2010; B. M. Nugent et al., 2015; Tsai et al., 2009; J. Xu, Deng, & Disteche, 2008). In addition, maternal care received by male and female rats differs, with males receiving more nurturing behaviors (i.e. licking) from dams in the home cage (C. L. Moore & Morelli, 1979; Richmond & Sachs, 1984). Also, at least in our maltreatment paradigm, females are subject to more adverse behaviors from dams

(Keller et al., 2019). Thus, the epigenetic changes at *Bdnf* may be more robust in maltreated females and thus require more robust intervention. Pinpointing an effective dose in females will be important to potentially address not only the behavioral deficits reported for females in Chapter 2, but in investigating previously reported transgenerational effects of maltreatment on maternal behavior (Roth et al., 2009)

Given that systemic drug administration has global implications, global measurement of its effects are necessary. While Chapter 3 reports no drug-induced differences in global methylation levels, it is entirely possible that bidirectional methylation changes in different genes are obscuring global differences. Thus, future work will require a more in-depth global examination that reveals gene-specific changes outside of *Bdnf*. It bears repeating here that data suggest only a portion of genes are subject to the effects of HDACi drugs (Weaver et al., 2006; W. S. Xu et al., 2007), though the mechanisms of this specificity are poorly understood. As this work progresses it will also be important to measure changes in expression induced by altered methylation levels, particularly considering that increases in methylation are sometimes associated with increased gene expression (Chahrour et al., 2008).

It will also be necessary to further examine off-target drug effects. The current work takes a small step in this direction via assessment of a *Bdnf* exon (exon IV) known to be unaffected by maltreatment in the infant PFC. As reported, this exon remained unaffected in maltreated animals given sodium butyrate. In addition, neither exon IV nor IX methylation levels were affected by sodium butyrate in control animals. However, HDACi drugs can act on multiple substrates, affecting multiple processes (Gagliano et al., 2014; Kruh, 1981), meaning any study of their effect on

behavior will need to examine off-target effects and their implications. Our lab is currently assessing off-target drug effects in other, ongoing experiments.

Overall, the current data provide a strong starting point for better understanding how epigenetic alterations contribute to altered behavioral trajectories following disruption of the caregiving relationship. Chapter 2 reports male-specific behavioral deficits following maltreatment and Chapter 3 reports male-specific prevention of maltreatment-induced *Bdnf* methylation in the PFC. These data give us the information we need to move forward and examine whether this prevention extends to the adult male brain and if it does, what that means in terms of normalizing male behavior following maltreatment. These data have also provided us with more information to answer these questions in maltreated females. Overall, the data reported here represent a step forward in understanding epigenetic and behavioral landscapes as they are shaped by exposure to adversity during development.

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Appendix A

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL FORMS

University of Delaware
Institutional Animal Care and Use Committee
Annual Review

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Title of Protocol: Epigenetic mechanisms in lifelong changes in genes and behavior associated with adverse caregiving	
AUP Number: 1216-2015-1	← **(4 digits only)**
Principal Investigator: Tania L. Roth	
Common Name: Rat (Long Evans Blue Spruce) **Genus Species:** Rattus norvegicus	

Pain Category: ***(please mark one)***

USDA PAIN CATEGORY: ***(Note change of categories from previous form)***	
Category	**Description**
☐ **B**	Breeding or holding where NO research is conducted
☐ **C**	Procedure involving momentary or no pain or distress
☐ **D**	Procedure where pain or distress is alleviated by appropriate means (analgesics, tranquilizers, euthanasia etc.)
☒ **E**	Procedure where pain or distress cannot be alleviated, as this would adversely affect the procedures, results or interpretation

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IACUC Approval Signature: Glenn Tellier, DVM

Date of Approval: 2/1/15

University of Delaware
Institutional Animal Care and Use Committee
Annual Review

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Title of Protocol: Epigenetic mechanisms in lifelong changes in genes and behavior associated with adverse caregiving

AUP Number: 1216-2016-2 ← **(4 digits only)**

Principal Investigator: Tania L. Roth

Common Name: Rat (Long Evans Blue Spruce)

Genus Species: Rattus norvegicus

Pain Category: ***(please mark one)***

USDA PAIN CATEGORY: ***(Note change of categories from previous form)***

Category	Description
☐ B	Breeding or holding where NO research is conducted
☐ C	Procedure involving momentary or no pain or distress
☐ D	Procedure where pain or distress is alleviated by appropriate means (analgesics, tranquilizers, euthanasia etc.)
☒ E	Procedure where pain or distress cannot be alleviated, as this would adversely affect the procedures, results or interpretation

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IACUC Approval Signature: Glenn Tatham, DVM

Date of Approval: 2/1/16

University of Delaware
Institutional Animal Care and Use Committee
Application to Use Animals in Application to use animals in Research
(New and 3-Yr submission)

Title of Protocol: Epigenetic mechanisms in lifelong changes in genes and behavior associated with adverse caregiving	
AUP Number: 1216-2017-0	**← (4 digits only — if new, leave blank)**
Principal Investigator: Tania L. Roth	
Common Name (Strain/Breed if Appropriate): Rat (Long Evans Blue Spruce) **Genus Species:** Rattus norvegicus	
Date of Submission: 12-19-16	

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IACUC Approval Signature: Tania Roth, DVM

Date of Approval: 1/30/2017

Appendix B

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