

**THE IMPACT OF AVERSIVE CAREGIVING
ON DNA METHYLATION IN BLOOD
AND THE PREFRONTAL CORTEX**

by

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ON DNA METHYLATION IN BLOOD
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ABSTRACT

Aversive caregiving causes epigenetic alterations that may lead to neurological and behavioral problems later in life. These issues presumably originate in the brain, and, as such, epigenetic changes within the brain would appear to be the most relevant to better understand the origin of and possible alleviation of these issues. However, it is difficult to study epigenetics in the brain in living humans, so many researchers rely on epigenetic alterations in peripheral tissues to be an indication of aberrant epigenetic mechanisms within the brain. The reliability of using such epigenetic changes in the periphery as a proxy for that of the brain is still largely unknown. Rodent models provide a much-needed mechanism of testing whether epigenetic alterations in peripheral tissues like blood mirror that of the brain. The current study used a rodent model to examine changes in DNA methylation at two regions (exons IV and IX) of the *Brain-derived neurotrophic factor (Bdnf)* gene in both the prefrontal cortex and blood following aversive caregiving. DNA methylation increased in response to aversive caregiving at exons IV and IX in both the prefrontal cortex and blood. Despite the similar group trends in both tissues in response to aversive caregiving, no correlations were found between DNA methylation in the brain and the blood. Data overall indicate that *Bdnf* DNA methylation in the blood and brain tissue is similarly responsive to early-life experiences, but the correlational analyses suggest that this relationship varies at the individual subject level.

Chapter 1

INTRODUCTION

1.1 Aversive Caregiving

Child maltreatment is a prevalent issue in today's society. It is defined as any kind of neglect or abuse inflicted on minors and includes but is not limited to negligence and physical, sexual, or emotional abuse (World Health Organization, 2016). According to the World Health Organization (2016), one in four adults report physical abuse in their childhood, and The U.S. Department of Health and Human Services (2018) found that over 7.4 million children had been involved in reported incidents of child maltreatment within the United States in 2016. Of these maltreated children, 1,750 were killed due to abuse or neglect (U.S. Department of Health and Human Services, Administration for Children and Families, Administration on Children, Youth and Families, Children's Bureau, 2018).

The percentage of incidents that are fatal may seem low at just 0.02% (U.S. Department of Health and Human Services, Administration for Children and Families, Administration on Children, Youth and Families, Children's Bureau, 2018), but those who survive child maltreatment are not necessarily free from all repercussions. These childhood incidents may have a long-lasting effect on the victims' mental and physical health. This kind of early-life adversity can be associated with changes in brain structure, changes in the stress response system, and social and cognitive deficits later in life (De Bellis, 2005; Gould et al., 2012). There is also a relationship between child maltreatment and later-life psychopathology, including depression (Gibb, Butler, &

Beck, 2003; Li, D'Arcy, & Meng, 2016; Widom, DuMont, & Czaja, 2007), anxiety (Gibb, et al., 2003; Li, et al., 2016), bipolar disorder (Kefeli, Turow, Yildirim, & Boysan, 2018), and schizophrenia (Bahari-Javan et al., 2017). The link between aversive caregiving and later consequences such as these could be through a mechanism known as epigenetics.

1.2 Epigenetics

The term “epigenetics” was first coined in 1942 by Conrad H. Waddington, who used the term to refer to the driving relationship between genes and their products (reviewed in Goldberg, Allis, & Bernstein, 2007). It was initially used as a catch-all for features outside of the realm of understood genetic principles (Goldberg et al., 2007). Since this first instance, the definition has been subtly changed many times over. Recently, it has been defined as the context-dependent activation and deactivation of genes (Moore, 2015). Modern definitions such as this typically suggest that epigenetics is the altered expression of genetic material due to one’s environment.

The prefix “epi” means “above” or “upon,” meaning the literal translation of “epigenetics” is “above” or “upon” genetics. Years after Waddington coined the term, it was discovered that epigenetic factors could bind to DNA or to the proteins associated with it, such that they are in fact literally “above” or “upon” the genetic code. These molecules can then either increase or decrease the expression of a gene or genes. Epigenetics does not refer to changes in the genome itself, but rather changes to the DNA structure and how those changes may alter gene expression

1.2.1 Histone Acetylation

One mechanism through which epigenetics can take place is histone acetylation. DNA is wrapped tightly around proteins called histones, which have N-terminals known as histone tails (reviewed in Eberharter & Becker, 2002). Certain molecules, including acetyl groups, can bind to these histone tails and increase the expression of nearby genes (reviewed in Audia and Campbell, 2016; reviewed in Eberharter & Becker, 2002). Histone acetyltransferases (HATs) can transfer acetyl groups to histones, resulting in gene expression, while histone deacetylases (HDACs) can remove acetyl groups, resulting in gene silencing (reviewed in Bannister and Kouzarides, 2011; reviewed in Eberharter & Becker, 2002). Histone acetylation provides a looser style of chromosomal packing, making the DNA more accessible and allowing transcription to take place (reviewed in Eberharter & Becker, 2002).

1.2.2 DNA Methylation

Another epigenetic mechanism is DNA methylation. This refers to the addition of a methyl group to cytosines. DNA methyltransferases (DNMTs) remove a methyl group from S-adenyl methionine (SAM) and place the methyl group onto the fifth carbon of a cytosine, which then becomes 5-methylcytosine (5mC) (reviewed in Moore, Le, & Fan, 2013). Often, DNA methylation occurs at cytosine-guanine dinucleotides known as CpG sites (reviewed in Moore et al., 2013).

DNA methylation in which an unmethylated region becomes methylated is known as *de novo* DNA methylation and usually involves DNMT3a or DNMT3b (reviewed in Moore et al., 2013). DNA methylation also occurs during DNA replication. The copying over of DNA methylation to the daughter strand is known as maintenance DNA methylation and involves DNMT1 (reviewed in Moore et al.,

2013). Just as cytosines become methylated, they can also become demethylated. Passive DNA demethylation results during DNA replication when the DNA methylation is simply not copied over to the daughter strand (reviewed in Moore et al., 2013). Improper function of DNMT1 could result in this process (reviewed in Moore et al., 2013). Although a direct DNA demethylation molecule has yet to be discovered, active DNA demethylation can occur through a series of chemical reactions that modify 5mC (reviewed in Moore et al., 2013).

DNA methylation is most often associated with gene silencing. This occurs partly because the methyl groups act as a physical barrier for transcription factors that must interact with the DNA before it can be transcribed and expressed as a protein (reviewed in Moore et al., 2013). DNA methylation also attracts methyl-CpG-binding domain (MBD) proteins (reviewed in Moore et al., 2013). These MBD proteins can then attract corepressors, such as histone deacetylase (HDAC) (reviewed in Moore et al., 2013). HDACs can remove acetyl groups from histones, thus decreasing gene expression (reviewed in Eberharter & Becker, 2002). Although most of the literature is consistent with DNA methylation leading to decreased gene expression, it has occasionally been associated with gene activation. In this case, methyl-binding proteins may recruit co-activators that lead to histone acetylation, and thus, an increase in gene expression (Chahrour et al., 2008).

1.2.3 The Importance of Epigenetics

Epigenetics has enormous relevance to health concerns. Indeed, epigenetic alterations are present in disorders such as schizophrenia (Dempster et al., 2011; Grayson et al., 2005), bipolar disorder (D'Addario et al., 2012; Dempster et al., 2011), and depression (Chagnon, Potvin, Hudon, & Prévaille, 2015; reviewed in Chen, Meng,

Pei, Zheng, & Leng, 2017; Fuchikami et al., 2011; Haghighi et al., 2014). Epigenetic changes that are associated with the risk, onset, outcome, or therapeutic response of a disorder are known as biomarkers, and they can be important in diagnosing patients and developing treatment methods (Bakulski, Halladay, Hu, Mill, & Fallin, 2016; García-Giménez et al., 2017). Whether these epigenetic mechanisms cause the disorders, cause certain symptoms of the disorders, or occur as non-etiological byproducts of the disorders is not yet fully understood, but further research into these biomarkers will likely improve our understanding of neural and behavioral pathology, as well as targets of potential treatment for these disorders.

Epigenetics also explains how nature and nurture can impact development. In addition to genetic inheritance, the environment can have widespread impacts on the body. It is unknown quite how environmental experiences may trigger enzymes like HATs and DNMTs to produce epigenetic modifications, but many studies do show relationships between environmental experiences and epigenetic alterations. For example, research findings highlight a role for diet in epigenetic changes. One study found differences in coat color of agouti mice offspring between two different diets, one with methyl donors and one without (Waterland & Jirtle, 2003). Another study found that those who were prenatally exposed to the Dutch famine had significantly less methylation of a gene involved in growth and development than their siblings who were not exposed to the famine (Heijmans et al., 2008). Additionally, studies have found relationships between epigenetic changes and exercise (Abel and Rissman, 2013; Brown, 2015; Gomez-Pinilla, Zhuang, Feng, Ying, & Fan, 2011; Lindholm et al., 2014), smoking (Harlid, Xu, Panduri, Sandler, & Taylor, 2014), drug abuse (reviewed in Nestler, 2014), and parenting styles (Beach, Lei, Brody, Dogan, &

Philibert, 2015). These findings provide evidence that even seemingly small environmental exposures have a great and long-term impact on changes to an individual's epigenome.

1.3 A Rodent Model for Aversive Caregiving

Both human and non-human animal research have examined the relationship between early-life adversity and epigenetic changes. Human research, although valuable, must be performed and examined with caution. It is difficult to limit variables and determine causation in human studies due to the vast complexity and diversity of human life. Research with non-human animals can be much more precisely controlled. Additionally, while human research may be more immediately relevant, rodent models are able to approach an argument for causation more readily, and as such, have resulted in many valuable findings with direct translational relevance to the human condition.

One of the leading papers examining aversive caregiving explored the relationship between maternal behavior and DNA methylation of the *GR* (glucocorticoid receptor) gene, which plays an important role in the stress response system (De Kloet, Vreugdenhil, Oitzl, & Joëls, 1998). This study found that low levels of maternal behavior (measured by frequency of licking, grooming, and arched-back nursing) corresponded to increased DNA methylation and decreased expression of the *GR* gene in the brain (Weaver et al., 2004). As a result, they conclude that pups who do not receive sufficient maternal care may be ill-suited to handle stress later in life. Indeed, these rats produce more corticosterone, and therefore, have an exaggerated stress response compared to control rats (Weaver et al., 2004). This increase in *GR* methylation has been replicated in humans who were abused in childhood and had

since committed suicide (McGowan et al., 2009). Other research has shown that pups who experienced maternal separation early in life exhibit both epigenetic and behavioral changes later in life, and these changes may even be passed on to future generations (Franklin et al., 2010).

Our lab has been most focused on how aversive caregiving impacts methylation of the brain-derived neurotrophic factor (*Bdnf*) gene. In addition to being involved in disorders such as depression, bipolar disorder, and schizophrenia, *Bdnf* is known to play a role in brain development, neuroplasticity, neurogenesis, synaptic transmission, and learning and memory (Binder & Scharfman, 2004; Zheleznyakova, Cao, & Schliöth, 2016). The rodent *Bdnf* gene is rather complex, containing nine 5' non-coding exons, each linked to a unique promoter that splices to the common 3' coding exon IX (Fig. 1) (Zheleznyakova et al., 2016; Zheng, Zhou, Moon, & Wang, 2012). DNA methylation can occur at multiple exons within the *Bdnf* gene, thus often altering gene expression (Zheleznyakova et al., 2016, Zheng et al., 2012).

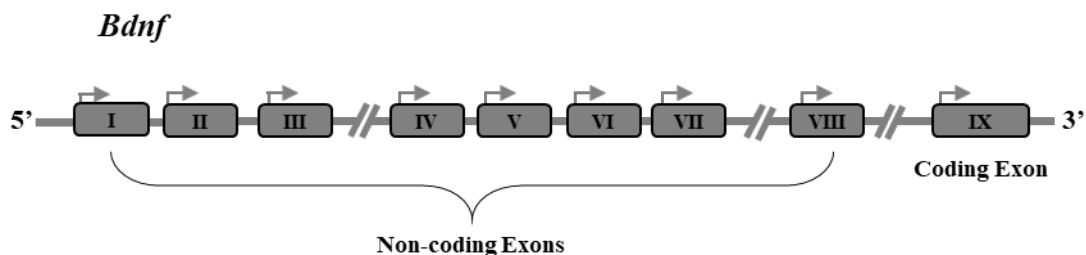


Figure 1. Schematic of the rodent *Bdnf* gene.

Our lab has used a rodent caregiver model to examine how pups who receive more aversive and less nurturing treatment may differ in terms of their epigenome and

behavior. Aversive caregiving corresponds with increased DNA methylation and decreased expression of the *Bdnf* gene, particularly at exon IX, in the prefrontal cortex (PFC) (Roth, Lubin, Funk & Sweatt, 2009). A similar effect was also seen in the PFC at exon IV in adulthood (PN90), but not in infancy (PN8) or adolescence (PN30) (Roth et al., 2009). This rodent model not only produces changes in the brain, but also in behavior. Pups who received more aversive care had changes in forced swim behavior, novel object recognition, cued-fear conditioning, and maternal behavior (Doherty, Blaze, Keller, & Roth, 2017).

Although this research is not performed in humans, animal studies are crucial to scientific discoveries and advancement. Such data provide empirical support that early-life adversity can cause long-lasting biological changes. Rodent models have also been successful in using treatment methods to reverse such changes (Roth et al., 2009; Weaver et al., 2004). This paves the way for determining the effectiveness of these treatments on humans with related biological or behavioral problems.

1.4 Peripheral Tissue

Human studies can generally only access the brain in postmortem cases. These studies tend to be less controlled, as it is difficult to determine or control for all relevant aspects of the deceased's life (e.g. life experiences, diet, drugs, etc.). Since postmortem samples come from a considerably limited pool, it can also be difficult to obtain desired sample types. It is also possible that death itself causes epigenetic changes unbeknownst to the experimenters (Bakulski et al., 2016). Additionally, since aging is known to be correlated with DNA methylation, the tendency for the postmortem samples of healthy controls to be older could be biasing results (Bakulski et al., 2016). Furthermore, because sample collection cannot take place at different

time points, postmortem samples do not allow for longitudinal studies, a useful experimental design strategy when attempting to understand causation and changes across time (Bakulski et al., 2016). Although postmortem studies are valuable in their own right, it can be more convenient and effective to perform studies on living participants. Therefore, many studies explore the epigenome within peripheral tissues, such as saliva, buccal cells, and blood.

The hope is that some epigenetic changes detected in these peripheral tissues are potential biomarkers, reflective of similar processes in the brain tissue, that can be collected and examined in a relatively noninvasive manner. A recent study found a significant increase in DNA methylation of *Bdnf* exon IV in the saliva of adults with borderline personality disorder compared to controls but did not find such a difference in blood (Thomas et al., 2018). Another study found that in adults with borderline personality disorder, *Bdnf* DNA methylation at exons I and IV in the blood increased with increasing reports of childhood trauma (Perroud et al., 2013). Increased *Bdnf* DNA methylation at exon VI in blood has also been reported in adult reporting low maternal care compared to high maternal care (Unternaehrer et al., 2015). When examining a region of the *BDNF* gene that includes the exon IV promoter in whole blood cells, women with Bulimia Nervosa and a history of physical abuse in childhood were found to have a significantly higher percentage of methylation than healthy controls (Thaler et al., 2014). Additionally, children who were previously maltreated had significantly higher methylation levels of the *GR* gene compared to controls (Romens, McDonald, Svaren, & Pollak, 2015).

Possible biomarkers in the periphery have also been found for depression. *Bdnf* DNA methylation is higher within the saliva of women with anxiety or depression

compared to age-matched controls (Chagnon et al., 2015). Additionally, *Bdnf* exon I has significantly different DNA methylation in the blood of adults with major depression (Fuchikami et al., 2011). Researchers have also investigated biomarkers for bipolar disorder. In blood, *Bdnf* DNA methylation is increased in adults with bipolar disorder type II compared to healthy controls (D'Addario et al., 2012). Biomarkers such as these can not only be used to aid diagnoses, but also in determining the root origin of certain symptoms and in finding treatments or even cures.

Noninvasive biomarkers of disease are not the only reason to investigate the epigenome of peripheral tissue. While many human studies examine peripheral tissue samples, researchers are often most interested in epigenetic changes within the brain, because this is presumably where many behavioral changes and psychopathologies originate. Hence, researchers attempt to use the epigenome within saliva, buccal cells, or blood as proxies for that of the brain. Blood is a particularly common peripheral tissue for analysis, because blood draws are often already required of patients and thus blood is accessible to many researchers (Bakulski et al., 2016). If it is an accurate estimation, the epigenetic changes in blood could ultimately be used to noninvasively examine epigenetic changes in the brain.

1.5 The Correlation Between Tissues

Despite the dependence on peripheral tissue to estimate epigenetic changes within the human brain, the correlation between epigenetic markers across tissues is still largely unknown. Several studies have examined this correlation, but due to the large scope of tissue types, sites of epigenetic changes, and causes for such epigenetic changes, there has been no decisive conclusion. Thus, it is important to continue to

examine this relationship to understand just what peripheral tissues do or do not inform us about the brain.

Some research has found correlations between DNA methylation in peripheral tissues and the brain, but not necessarily in blood. One study examining DNA methylation among saliva, blood, and the brain found a correlation between overall DNA methylation in saliva and blood, but found that the DNA methylation in saliva more closely resembled that of the brain, specifically, the cerebellum, frontal cortex, entorhinal cortex, and superior temporal gyrus (Smith et al., 2015). Furthermore, a correlation was found between *Bdnf* exon I DNA methylation in the ventral prefrontal cortex (vPFC) and quadriceps tissue, suggesting that some peripheral tissues do appear to be appropriate proxies for DNA methylation in the PFC (Stenz et al., 2015). Although the article did not investigate a correlation between brain and blood DNA methylation owing to the two tissues being collected from separate samples, an increase in DNA methylation at *BDNF* promoter I in males compared to females was observed across blood, quadriceps, and vPFC although at different CpG sites (Stenz et al., 2015).

There have also been some findings supporting a correlation between brain and blood DNA methylation. When investigating DNA methylation changes across age, strong correlations were found for average methylation between regions of the brain and blood while weaker, but still significant, correlations were found between the two tissues when examining age-related methylation changes (Horvath et al., 2012). Furthermore, in a mouse model for Cushing's disease, DNA methylation in the *Fkbp5* gene in the hippocampus and in blood showed a moderate correlation although the DNA methylation changes were at different CpG sites on different introns (Ewald et

al., 2014). A study examining DNA methylation of the *COMT* gene, which plays a role in stress response and working memory, found that *COMT* DNA methylation in the blood of rats was moderately correlated with that of the PFC but not significantly correlated with that of their hippocampi or striatum (Ursini et al., 2011). When Bisphenol A (BPA) exposure was used as a model for early-life adversity, the same patterns of sex-specific DNA methylation changes were found in both the hippocampus and in blood at *Bdnf* exon IV in adult rodents (Kundakovic et al., 2015). Regardless of exposure to BPA, the same study found a moderate correlation between the hippocampus and the blood at CpG site 4 *Bdnf* IV methylation (Kundakovic et al., 2015). Researchers have even developed a program that attempts to analyze DNA methylation changes in the blood in order to provide a representation for DNA methylation changes in the brain (Edgar, Jones, Meaney, Turecki, & Kobor, 2017).

Other studies have found evidence to suggest that DNA methylation in blood may not always be an appropriate proxy for that of the brain. One such study found that less than 8% of CpG sites across the entire genome showed strong correlations ($p \geq 0.59$) between the temporal lobe and blood (Walton et al., 2016). Similarly, a recent study found that less than 11% of individual CpG sites had correlations greater than $r = 0.5$ between the brain and blood (a higher proportion than between saliva and the brain or buccal cells and the brain) (Braun et al., 2019). While the study did find a strong correlation between global methylation in the brain and blood ($r = 0.86$), there was a significantly stronger correlation between saliva and the brain ($r = 0.90$) (Braun et al., 2019). Similar patterns were discovered when examining certain genes implicated in neurological disorders, one of which was *BDNF* (Braun et al., 2019). Additionally, only a trending correlation between DNA methylation of the *GR* gene in

white blood cells and the prefrontal cortex was found after stress (Witzmann, Turner, Mériaux, Meijer, & Muller, 2012). Another study found significant differences in DNA methylation patterns in the blood compared to regions of the cortex, and overall, greater differences between tissues than between individuals (Davies et al., 2012). However, DNA methylation differences found between individuals did show a strong correlation between the cortex and blood, suggesting some degree of intra-individual similarities across tissues (Davies et al., 2012). This finding suggests that DNA methylation changes that occur as a result of one's experiences, and thus vary between individuals, may indeed be correlated between the brain and blood. Additionally, one study reported vast differences in global DNA methylation between the cortex and blood despite strong correlations between the two in some select locations of the genome (Hannon, Lunnon, Schalkwyk, & Mill, 2015). Although this research does not eliminate the possibility of using blood as a proxy for the brain at certain regions of the genome, it does encourage caution in assuming that there will always be a correlation between the two tissues.

There seems to be some evidence for a correlation between DNA methylation in the brain and blood, particularly for those studies examining DNA methylation changes in a specific gene as a result of a disorder, stressor, or other factor (Ewald et al., 2014; Kundakovic et al., 2015; Ursini et al., 2011). While there are many researchers working to examine the relationship between brain and blood DNA methylation, there is still much to be explored. It seems that although DNA methylation is not precisely the same between these tissues, there may be a correlation between the two when examining DNA methylation in a certain gene brought about by external factors or disorders.

It is largely unknown why these tissues may or may not exhibit the same DNA methylation changes. The brain develops from the ectodermic layer during differentiation while blood develops from the mesoderm, thus the cells have very different lineages (Davies et al., 2012). Different brain regions then develop separately as they begin to distinguish themselves as distinct functioning parts of a cohesive neural system (Davies et al., 2012). Due to these functional and developmental distinctions between a peripheral tissue like blood and cortical areas, it would appropriately follow that throughout development, DNA methylation patterns would differ. Key epigenetic mechanisms originating earlier in development would presumably be more widely spread throughout the body. However, DNA methylation changes that occur not as a result of development, but as a result of external factors such as stress may or may not follow this pattern. Perhaps some external factors cause epigenetic changes throughout the body while others are more targeted. Peripheral biomarkers for neuropathological disorders would suggest that the same epigenetic factors impacting the brain are also creating changes in the periphery. However, the extent and specifics of this phenomenon and the reason behind it are still very much up for debate, and as such, require further investigation.

1.6 Rationale for Current Research

The current research utilized a rodent model and aimed to explore the relationship between brain and blood DNA methylation with respect to aversive caregiving. This experiment used the scarcity-adversity model of low nesting resources outside the home cage, in which rat pups from an experimental litter were divided into three groups. For 30 minutes each day, for the first seven days of life, one-third of the litter were placed in the care of another dam who was in a stressful

environment and was likely to mistreat the pups. Another third were placed in the care of another dam who was in a non-stressful environment and was likely to nurture the pups. The final third remained with their biological mother after being weighed and marked for identification each day. The latter two groups served as controls for moving or not moving the pups to a new dam, in the absence of maltreatment.

Our lab has previously explored how this paradigm impacts *Bdnf* DNA methylation at exons IV and IX in the PFC (Roth et al., 2009) but not in blood. DNA methylation can be aberrant at exons IV and IX in both rodents and humans in response to various stressors and other factors (Doherty, Chajes, Reich, Duffy, & Roth, 2019; Hsieh, Lin, Lee, & Huang, 2019; Kundakovic et al., 2015; Perroud et al., 2013; Roth et al., 2009; Thaler et al., 2014; Thomas et al., 2018). Exon IV and IX transcripts are the two most plentiful in blood in humans (Cattaneo, Cattane, Begni, Pariante, & Riva, 2016), and although limited, the rodent literature suggests a similar trend (Aid, Kazantseva, Piirsoo, Palm, & Timmusk, 2007). As such, these two exons seemed to be good targets for an initial study in the blood. The current research explored DNA methylation in *Bdnf* exons IV and IX at PN8 in both the PFC and blood. A subset of the animals underwent perfusions, consisting of a saline flush, prior to brain extractions in order to rid the PFC of blood that could be influencing methylation in this region.

As there is very little blood in the immature cortex (fetal lamb cortices are estimated to have 3 milliliters of whole blood per 100 grams of brain weight (Barfield et al., 1999)), it was not expected that the perfusion and non-perfusion groups would display significantly different methylation patterns. However, increased methylation in

animals who were not perfused would be an interesting finding, as it would indicate that the remaining blood in the PFC samples can impact methylation results.

In the PFC, I predicted that *Bdnf* IX DNA methylation would be significantly greater in maltreated pups relative to control pups, in accordance with previous findings (Doherty et al., 2019; Phillips & Roth, unpublished data; Roth et al., 2009). I also predicted that *Bdnf* IV DNA methylation in the PFC would not be significantly different among the groups at PN8, as that change in DNA methylation has not previously been observed early in development (Doherty et al., 2019; Roth et al., 2009). In accordance with the human literature examining *Bdnf* methylation after child maltreatment or low maternal care (Perroud et al., 2013; Thaler et al., 2014; Unternaehrer et al., 2015), it was expected that the maltreatment group would show increased *Bdnf* methylation in the blood as well. If both tissues do indeed show increased methylation, it is expected that there will be a positive correlation between the two, at least at exon IX. This research was exploratory, and it was difficult to predict the results of the blood DNA methylation. Regardless of directional change or similarity between brain and blood DNA methylation, the outcome was predicted to be a valuable addition to the field.

Chapter 2

METHODS

2.1 Subjects

258 Long-Evans pups (128 male and 130 female) housed at the University of Delaware were used for measures of brain and blood DNA methylation in this study. One female and three males died prematurely and were, thus, not included in analysis. Additionally, two PFC samples and one blood sample were excluded due to product failure. Animals were kept in polypropylene cages (18"x9"x8") containing plenty of wooden shavings for use as bedding in a temperature- and light- controlled room. A 12-hour light/dark cycle (07:00-19:00) was used, performing all experimentation during the light cycle. Ample food and drink were accessible to the rats. Pups were generated from 25 dams, who were bred in-house and their first litters were not used to ensure that being a first-time mother did not impact the results. PN0 was designated as the day of birth, and litters were culled to 5-6 males and females each on PN1 when necessary. All practices were approved by the University of Delaware Animal Care and Use Committee prior to the project's beginning.

2.2 Caregiving Manipulations

The caregiver rodent model used in this experiment was previously used by this lab (Blaze, Scheuing, & Roth, 2013; Doherty et al., 2017; Doherty et al., 2019; Doherty, Forster, & Roth, 2016; Roth et al., 2009). The model consists of three conditions: normal maternal care, cross-foster care, and maltreatment (Fig. 2). Pups were randomly assigned to one of the three conditions with sexes balanced to the best of our ability. If necessary, two or three pups of the same sex, litter, and condition were averaged together for analysis to limit observable trends within a litter. Caregiver

manipulations took place for 30 minutes each day from PN1 to PN7. Those in the normal maternal care condition remained in the home cage with their biological dam. Those in the cross-foster care condition were placed with another dam in a chamber with ample bedding (about 2cm across the floor). This dam was given an hour in the chamber to habituate prior to the placement of the pups. Those in the maltreatment condition were placed with another dam who was given insufficient time to habituate and almost no bedding (only 100ml spread across the floor), thus limiting her ability to create a nest and provide nurturing behaviors. Previous research in our laboratory indicates that these conditions induce increased aversive behaviors compared to cross-foster and normal maternal care conditions (Blaze et al., 2013; Doherty et al., 2017; Roth et al. 2009).

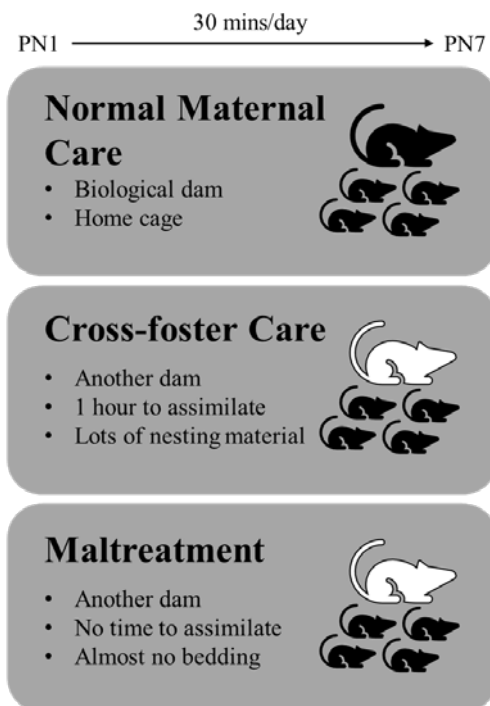


Figure 2. Schematic of the scarcity-adversity model of low nesting resources.

Maltreatment and cross-foster dams were lactating and matched to the biological dams by diet and postpartum age of their pups, as both factors can alter maternal behavior (Grieb, Holschbach, & Lonstein, 2018; Purcell et al., 2011). Both the cross-foster and the maltreatment conditions were run with white-noise in the background. Both of these conditions took place in the same room and in the same type of chamber: an 18"x12"x18" black, plastic container with a Plexiglas lid equipped with a red lightbulb. All pups, including those in the normal maternal care condition, were weighed and marked for identification purposes prior to these thirty minutes sessions. Both the normal maternal care and cross-foster care conditions served as controls. The pups belonging to the cross-foster care and maltreatment dams were kept in an incubator during the paradigm to ensure that a healthy body temperature was maintained. After the thirty minutes were up, all pups were returned to their home cages.

2.3 Behavioral Observations

Video cameras recorded the chambers during the 30-minute sessions for each of the three conditions. For seven litters, a trained observer scored each video for the dam's nurturing and aversive behaviors. Nurturing behaviors include pup licking, anogenital licking, hovering, and nursing while aversive behaviors include stepping, dragging, dropping, roughly handling, and actively avoiding the pups. Scorers indicated the occurrence of any of these behaviors for each of the 6 five-minute segments within the 30-minute sessions.

2.4 Tissue Collection, Perfusions, and DNA Methylation

Animals were euthanized at PN8. Animals in the perfusion group were anesthetized with 100ml/kg of a ketamine/xylazine solution (10ml ketamine to 1.5ml xylazine). A syringe was inserted into the left ventricle of the heart and saline (0.9% NaCl) was flushed through the body until the brain and internal organs were fully perfused. Blood samples for this group were taken directly from the left ventricle prior to perfusion while blood samples from the non-perfusion group were collected at the neck after decapitation. At least 100 microliters of blood were collected in tubes containing 50 microliters of EDTA. These tubes were gently mixed and stored in a -20° Celsius freezer. The PFC was dissected, homogenized with RLT buffer and 2-mercaptoethanol, and stored in a -80° Celsius freezer.

DNA and RNA were extracted from PFC tissue according to the protocol provided by the Qiagen AllPrep DNA/RNA kit. DNA was extracted from blood according to the protocol provided by the Qiagen DNeasy Blood and Tissue kit. Once extracted, DNA and RNA were stored in a -80° Celsius freezer. DNA from both the PFC and blood was then bisulfite converted according to the Qiagen Epitect Bisulfite Kit. This served to convert unmethylated cytosines to uracil. Primers capable of detecting methylated cytosines and specific to *Bdnf* exons IV and IX were used in methylation-specific real-time polymerase chain reactions (PCR) (MSP) to amplify the bisulfite-converted DNA. To determine whether there was a significant difference in the fold change among the experimental conditions (maltreatment, cross-foster care, and normal maternal care) in methylated *Bdnf*, the comparative Ct method was used with *Tubulin* as a reference gene (Livak & Schmittgen, 2001; Pfaffl, 2001).

2.5 Statistical Analysis

One-way and two-way ANOVAs were used to assess maternal behavior, using Tukey's and Sidak's post-hoc tests, respectively, as needed. DNA methylation data were analyzed using two-way ANOVAs, the factors being infant condition (maltreatment and cross-foster care) and presence of a perfusion or infant condition and sex. To compare DNA methylation to normal controls, one-sample *t*-tests were also used with a theoretical mean of one indicating no difference compared to controls. Unpaired *t*-tests and Mann-Whitney tests (if there was not homogeneity of variance) were used to compare maltreatment and cross-foster conditions after collapsing sex and perfusions. Differences in fold change were also used to run linear regressions between brain and blood DNA methylation. In any case in which there was more than one sample per sex per condition from a given litter, samples were averaged together to avoid oversampling from that litter. The value of statistical significance was set at $p < 0.05$.

Chapter 3

RESULTS

3.1 Maternal Behavior

Frequency of both nurturing (hovering, pup licking, anogenital licking) and aversive (dropping, actively avoiding, roughly handling, stepping, dragging) maternal behaviors were scored for seven litters to ensure that the aversive caregiving paradigm was effectively exposing the maltreatment group to a more aversive environment than the two control groups (Fig. 3). A 2-way ANOVA with the factors infant condition (normal care, cross-foster care, maltreatment) and behavior type (aversive, nurturing) revealed a main effect of behavior type ($F(1, 34) = 117.6, p < 0.001$) and an interaction ($F(2, 34) = 50.41, p < 0.001$), but no main effect of infant condition ($F(1, 34) = 0, p > 0.999$). Post-hoc analyses revealed that both normal care and cross-foster care groups experienced significantly more nurturing behaviors than aversive behaviors ($p < 0.001$; Fig. 3). One-way ANOVAs revealed main effects of infant condition for both abusive and nurturing behaviors ($F(2, 17) = 25.21, p < 0.001$). Post-hoc analyses showed significantly more abusive and less nurturing behaviors in the maltreatment condition compared to normal care ($p = 0.022$) and cross-foster care ($p < 0.001$) conditions. The cross-foster care condition also experienced significantly less abusive and more nurturing ($p = 0.015$) behaviors compared to normal maternal care, an unexpected finding based on previous data from the lab (i.e. we typically do not see cross-foster dams be more nurturing than control dams).

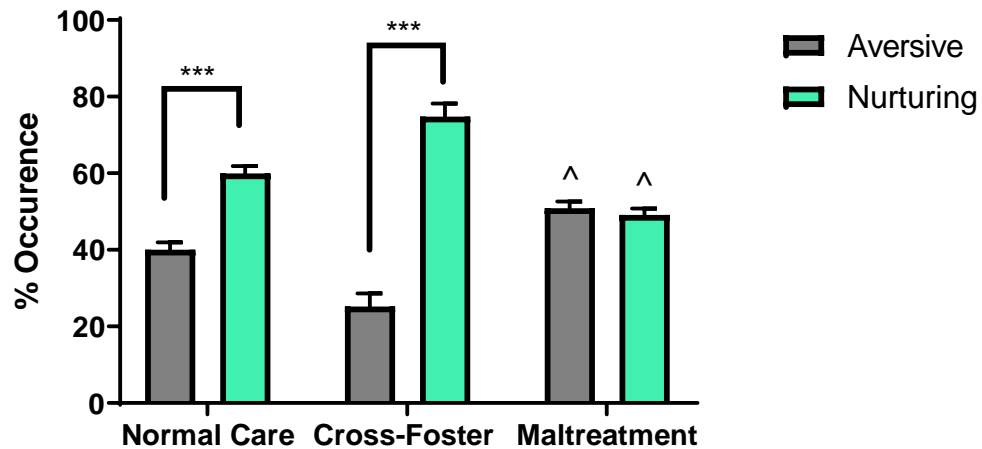


Figure 3. Percent occurrence of aversive and nurturing behaviors across the three infant conditions (normal care, cross-foster care, and maltreatment). *** $p < 0.001$, ^ $p < 0.05$ compared to normal and cross-foster care; error bars represent SEM.

3.2 *Bdnf* DNA Methylation at Exon IV Increases After Aversive Caregiving

DNA methylation was measured at exon IV of the *Bdnf* gene in the PFC and blood of animals in the maltreatment, cross-foster care, and normal maternal care groups. In the PFC, two-way ANOVAs between infant condition (maltreatment and cross-foster care) and perfusion revealed no main effect of perfusion in males (infant condition: $F(1, 59) = 2.913$, $p = 0.093$; perfusion: $F(1, 59) = 0.578$, $p = 0.450$; interaction: $F(1, 59) = 0.594$, $p = 0.444$) or females (infant condition: $F(1, 61) = 0.771$, $p = 0.384$; perfusion: $F(1, 61) = 1.561$, $p = 0.216$; interaction: $F(1, 61) = 1.012$, $p = 0.318$), so the data was collapsed across perfusions. A two-way ANOVA between infant condition and sex revealed no main effect of sex ($F(1, 124) = 0.941$, $p = 0.334$), a trending effect of infant condition ($F(1, 124) = 3.75$, $p = 0.055$), and no interaction ($F(1, 124) = 0.101$, $p = 0.751$). One sample *t*-tests revealed a significant increase in maltreated males ($t(31) = 2.204$, $p = 0.035$) and females ($t(31) = 3.287$, $p = 0.003$).

compared to normal care controls (Fig. 4). Collapsing both perfusion and sex, as neither were significant, revealed a significant increase in maltreatment compared to normal care ($t(63) = 3.829, p < 0.001$) and a trending increase compared to cross-foster care ($t(126) = 1.924, p = 0.057$) (Fig. 4).

Exploring exon IV methylation in blood after aversive caregiving revealed similar results to that of the PFC. Since a two-way ANOVA between infant condition and perfusion revealed no main effect of perfusion in males (infant condition: $F(1, 59) = 0.415, p = 0.522$; perfusion: $F(1, 59) = 1.963, p = 0.166$; interaction: $F(1, 59) = 2.073, p = 0.155$) or females (infant condition: $F(1, 61) = 0.779, p = 0.381$; perfusion: $F(1, 61) = 0.035, p = 0.852$; interaction: $F(1, 61) = 0.159, p = 0.691$), this factor could again be collapsed. A two-way ANOVA between infant condition and sex revealed no main effect of infant condition ($F(1, 124) = 1.472, p = 0.227$), sex ($F(1, 124) = 0.001, p = 0.979$), or an interaction between the two ($F(1, 124) = 0.186, p = 0.667$). However, maltreatment males ($t(31) = 2.111, p = 0.043$) and females ($t(31) = 2.523, p = 0.017$) exhibited significantly increased methylation compared to normal maternal care in one-sample t -tests (Fig. 4). Collapsing both sex and perfusion revealed a significant increase in maltreatment compared to normal care ($t(63) = 3.207, p = 0.002$) but not compared to cross-foster care ($U = 1869, p = 0.396$) (Fig. 4).

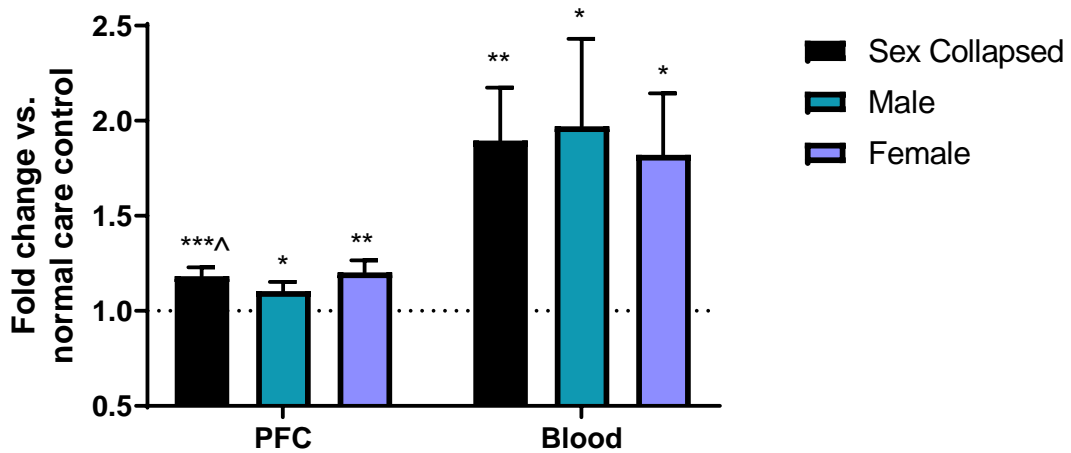


Figure 4. Fold change in *Bdnf* DNA methylation at exon IV in PFC and blood in the maltreatment group collapsed against perfusion type and graphed versus normal maternal care (dotted line). $n=12-21$ per group; * $p<0.05$ versus NMC; ** $p<0.01$ versus NMC; *** $p<0.001$ versus NMC; ^ $p<0.1$ versus CFC; error bars represent standard error of the mean.

3.3 *Bdnf* DNA Methylation at Exon IX Increases After Aversive Caregiving

Bdnf DNA methylation in PFC and blood was also examined at exon IX across the three infant conditions. In the PFC, perfusion groups could again be collapsed in both males (infant condition: $F(1, 59) = 0.422$, $p = 0.518$; perfusion: $F(1, 59) = 0.503$, $p = 0.481$; interaction: $F(1, 59) = 0.237$, $p = 0.628$) and females (infant condition: $F(1, 61) = 2.612$, $p = 0.111$; perfusion: $F(1, 61) = 0.049$, $p = 0.826$; interaction: $F(1, 61) = 0.838$, $p = 0.364$). A two-way ANOVA between infant condition and sex revealed no main effect of infant condition ($F(1, 124) = 2.638$, $p = 0.107$), sex ($F(1, 124) = 0.685$, $p = 0.410$), or an interaction ($F(1, 124) = 0.651$, $p = 0.421$), but maltreated males ($t(31) = 2.635$, $p = 0.013$) and females ($t(31) = 2.188$, $p = 0.036$) were both significantly increased compared to normal care controls in one-sample *t*-tests (Fig. 5). With sex and perfusion collapsed, the maltreatment group was significantly higher

than normal care ($t(63) = 3.044, p = 0.003$) and trending above cross-foster care ($U = 1665, p = 0.068$) (Fig. 5).

Exon IX methylation in the blood also followed a similar trend in maltreatment animals. Again, perfusion groups could be collapsed following two-way ANOVAs in males (infant condition: $F(1, 59) = 0.126, p = 0.723$; perfusion: $F(1, 59) = 1.015, p = 0.318$; interaction: $F(1, 59) = 2.250, p = 0.139$) and females (infant condition: $F(1, 61) = 1.082, p = 0.302$; perfusion: $F(1, 61) = 0.739, p = 0.394$; interaction: $F(1, 61) = 0.704, p = 0.405$). A two-way ANOVA between infant condition and sex then revealed no main effect of infant condition ($F(1, 124) = 1.142, p = 0.287$), sex ($F(1, 124) = 1.415, p = 0.237$), or an interaction ($F(1, 124) = 0.049, p = 0.825$). Following one-sample t-tests, maltreated males were not significantly higher than normal care ($t(31) = 1.590, p = 0.122$), but maltreated females were trending higher ($t(31) = 1.879, p = 0.070$) (Fig. 5). When both perfusion and sex were collapsed, the maltreatment group was significantly higher than normal care ($t(63) = 2.191, p = 0.032$), but not significantly different from cross-foster care ($t(126) = 1.049, p = 0.296$) (Fig. 5).

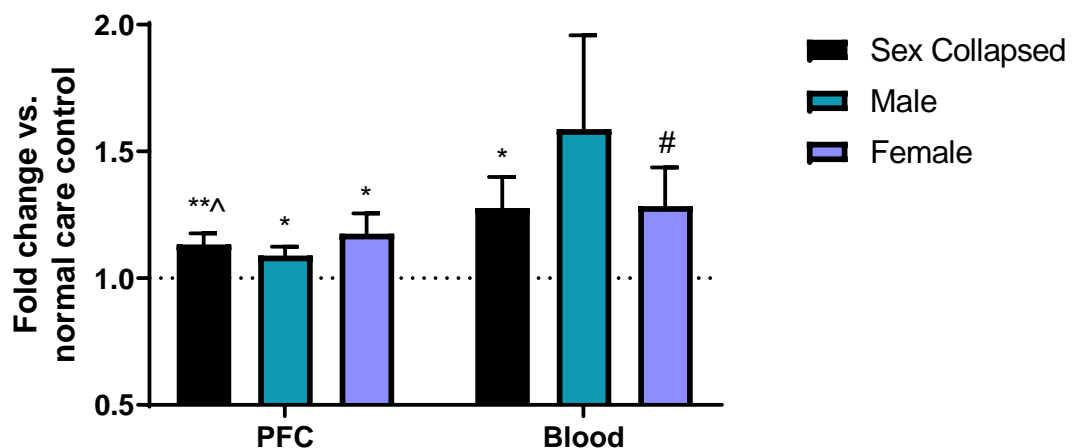


Figure 5. Fold change in *Bdnf* DNA methylation at exon IX in PFC and blood in the maltreatment group collapsed against perfusion type and graphed versus normal maternal care (dotted line). n=12-21 per group; *p<0.05 versus NMC; **p<0.01 versus NMC; #p<0.1 versus NMC; ^ p<0.1 versus CFC; error bars represent standard error of the mean.

3.4 *Bdnf* DNA Methylation in Blood, But Not PFC, Increases in the Cross-Foster Care Condition

As previously mentioned, there was no main effect of perfusion at either exon in either tissue, so the following results have been collapsed across perfusion groups. At exon IV in the PFC, no cross-foster care groups were significantly different from normal care (males: $t(30) = 0.303$, $p = 0.764$; females: $t(32) = 1.544$, $p = 0.133$; sex collapsed: $t(63) = 1.392$, $p = 0.169$). However, in blood, cross-foster females and the sex collapsed cross-foster group exhibited higher DNA methylation levels at exon IV than normal care ($t(32) = 2.507$, $p = 0.017$ & $t(63) = 2.934$, $p = 0.005$, respectively) (Fig. 6). Cross-foster males ($t(30) = 1.654$, $p = 0.109$) were not significantly different compared to normal care.

A similar trend is seen at exon IX. No significant differences were evident between cross-foster and normal care groups in the PFC (males: $t(30) = 1.087$, $p = 0.2858$; females: $t(32) = 1.181$, $p = 0.2464$; sex collapsed: $t(63) = 1.616$, $p = 0.1110$). However, in blood there were significant increases compared to normal care in cross-foster males and the sex collapsed cross-foster group ($t(30) = 2.445$, $p = 0.021$ & $t(63) = 3.166$, $p = 0.002$, respectively) (Fig. 6). There was a trending increase in cross-foster females (Fig. 6) ($t(32) = 2.016$, $p = 0.052$).

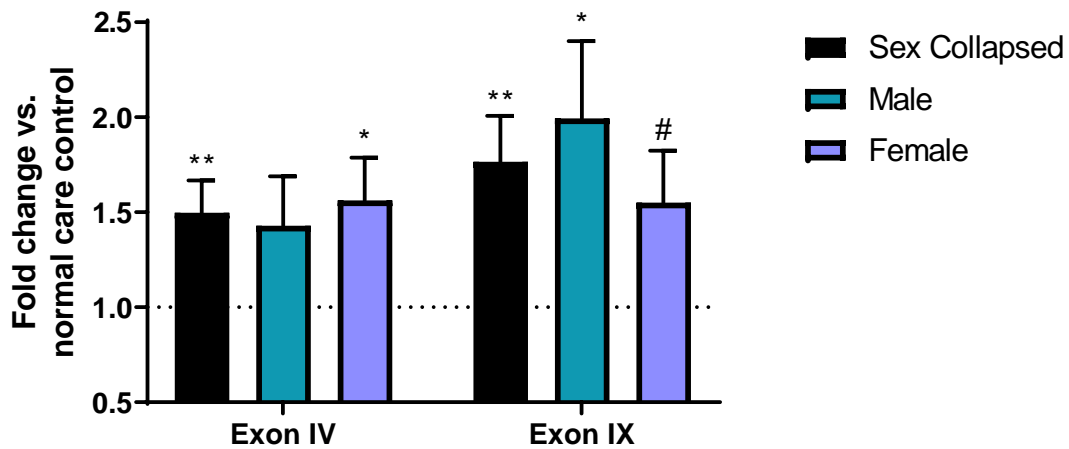


Figure 6. Fold change in *Bdnf* DNA methylation at exons IV and IX in blood in the cross-foster group collapsed against perfusion type and graphed versus normal maternal care (dotted line). n=12-21 per group; *p<0.05 versus NMC; **p<0.01 versus NMC; #p<0.1 versus NMC; error bars represent standard error of the mean.

3.5 No Correlations Between *Bdnf* DNA Methylation in the PFC or Blood

After collapsing across perfusions, linear regressions between PFC and blood *Bdnf* DNA methylation were run for any group that showed similar trends in both brain and blood. At exon IV, this included all maltreatment groups (sex collapsed, males, and females; Fig. 4) as well as cross-foster males (Fig. 6). At exon IX, this included maltreated females and the maltreatment sex collapsed group (Fig. 5).

Maltreated males ($r = -0.096$, $p = 0.600$), maltreated females ($r = 0.076$, $p = 0.678$), and the maltreatment, sex collapsed group ($r = -0.034$, $p = 0.792$) did not show significant correlations at exon IV (Fig. 7). Cross-foster males at exon IV also did not reveal a significant correlation between PFC and blood ($r = -0.077$, $p = 0.681$) (Fig. 8A, B). Similarly, there were no significant correlations at exon IX in maltreated

females ($r = -0.273$, $p = 0.131$) or the maltreatment group with sexes collapsed ($r = -0.094$, $p = 0.462$) (Fig. 8C-E).

49.7% of the data points in these correlations exhibited either increased DNA methylation in both PFC and blood or decreased methylation in both PFC and blood relative to normal care (blue points in Fig. 7 & 8, gray cells in Table 1). The other 50.3% of the data points showed an increase in methylation in one tissue and a decrease in the other (or one remained the same) relative to normal care.

Correlational analyses were also performed after log transforming the data and taking difference scores compared to the mean for each group in a similar vein to the analysis performed in Davies et al., 2012. Just as with the original correlational analyses, none of these correlations were significant (maltreated males at exon IV: $r = -0.213$, $p = 0.243$, maltreated females at exon IV: $r = 0.262$, $p = 0.147$, maltreatment group sex collapsed at exon IV: $r = 0.014$, $p = 0.915$, cross-foster males at exon IV: $r = -0.128$, $p = 0.493$, maltreated females at exon IX: $r = -0.111$, $p = 0.552$, maltreatment group sex collapsed at exon IX: $r = -0.218$, $p = 0.083$)

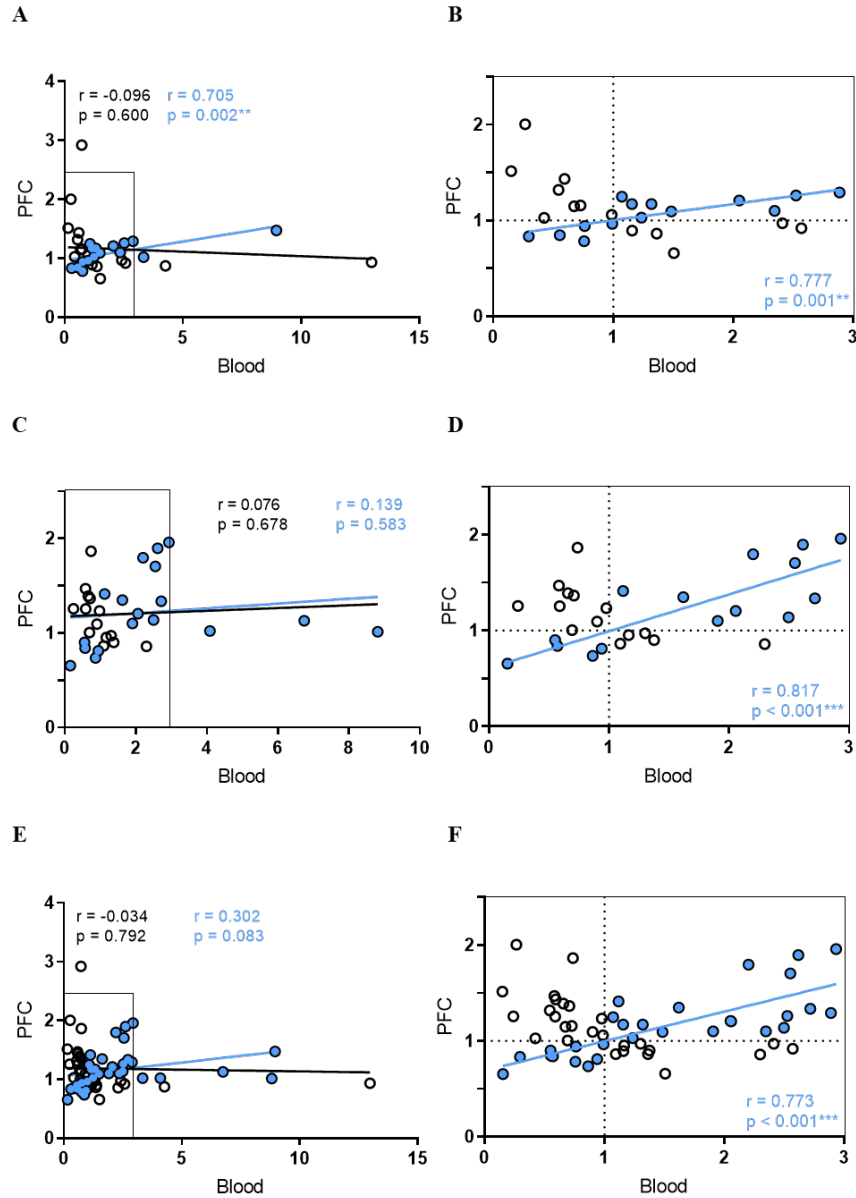


Figure 7. Correlations between PFC and blood DNA methylation at exon IV in (A, B) maltreated males, (C, D) maltreated females, and (E, F) maltreated animals collapsed across sex. DNA methylation was quantified in terms of fold change versus normal care (dotted lines). Blue points increased or decreased in both PFC and blood (both coordinates >1 or <1). White points did not follow the same trends between the two tissues. Black lines show the linear regression of all the data. Blue lines show the linear regression of the blue data points in each figure. B, D, and F show the subsets of the data in A, C, and E within the boxes. n = 12-21 per group.

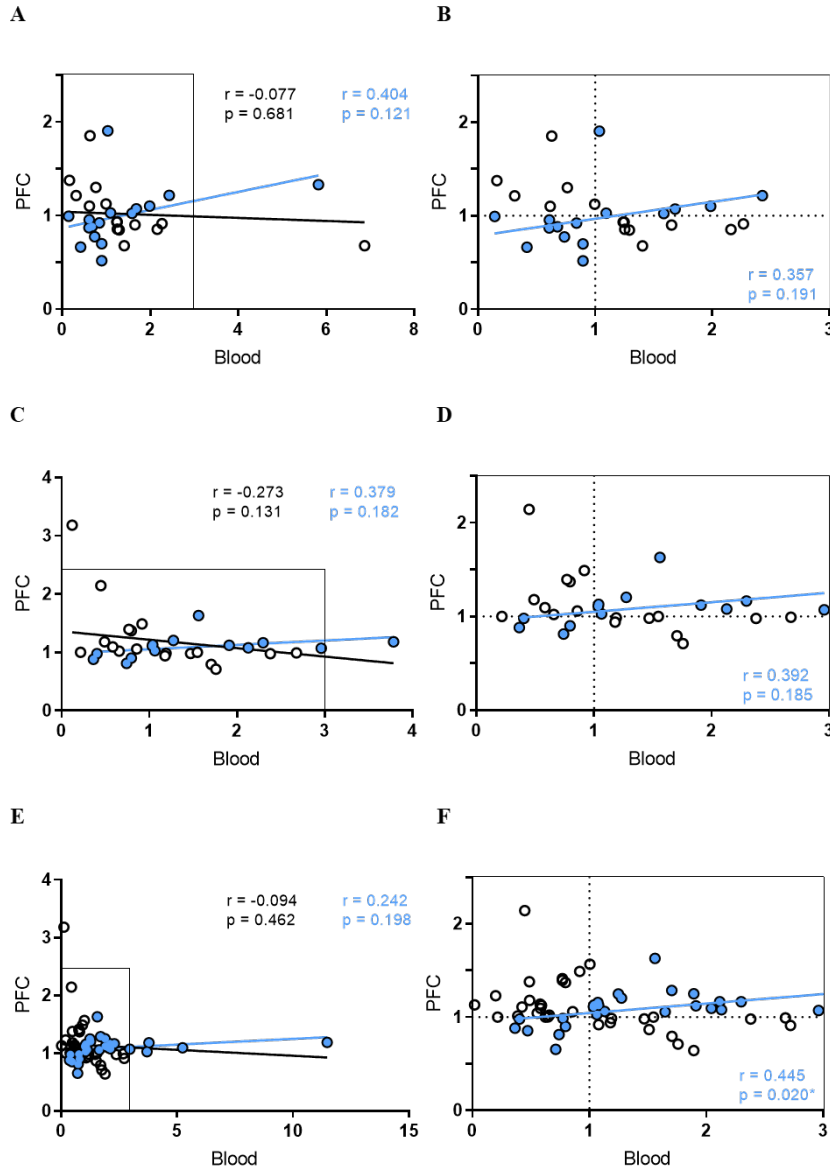


Figure 8. Correlations between PFC and blood DNA methylation in (A, B) cross-foster males at exon IV, (C, D) maltreated females at exon IX, and (E, F) maltreated animals collapsed across sex at exon IX. DNA methylation was quantified in terms of fold change versus normal care (dotted lines). Blue points increased or decreased in both PFC and blood (both coordinates >1 or <1). White points did not follow the same trends between the two tissues. Black lines show the linear regression of all the data. Blue lines show the linear regression of the blue data points in each figure. B, D, and F show the subsets of the data in A, C, and E within the boxes. $n = 12-21$ per group.

Table 1. Fold change in *Bdnf* DNA methylation in PFC and blood used for correlational analyses for maltreated males at exon IV, maltreated females at exon IV, cross-foster males at exon IV, maltreated males at exon IX, and maltreated females at exon IX. Gray cells indicate PFC/blood pairings that either both increased or both decreased in methylation relative to normal care. *Maltreated males at exon IX are included for the sake of examining the maltreatment group with sexes collapsed at exon IX.

MAL IV				CFC IV		MAL IX			
Males		Females		Males		Males*		Females	
PFC	Blood	PFC	Blood	PFC	Blood	PFC	Blood	PFC	Blood
0.87	4.26	1.13	6.74	0.86	1.25	1.06	1.13	0.98	1.47
1.47	8.96	1.10	1.91	1.33	5.82	1.11	0.42	0.80	1.71
2.01	0.27	1.39	0.66	0.96	0.61	1.57	1.00	1.13	1.04
1.06	0.99	1.01	0.69	1.12	1.00	0.86	0.47	1.12	1.91
0.92	2.57	1.96	2.93	0.92	0.84	1.01	0.39	1.07	2.96
0.94	12.98	1.37	0.71	0.77	0.74	1.29	1.70	1.18	0.49
1.26	2.52	1.34	2.72	1.30	0.76	1.25	1.89	0.71	1.76
0.86	1.36	1.47	0.58	1.02	1.59	1.00	0.65	0.81	0.74
1.21	2.05	1.80	2.20	1.21	2.43	1.38	0.49	1.37	0.80
1.03	1.24	1.35	1.62	1.03	1.09	0.91	2.72	1.63	1.56
1.29	2.89	1.21	2.06	0.52	0.90	1.13	0.02	0.99	1.19
0.94	0.76	0.74	0.86	0.93	1.24	0.64	1.89	2.14	0.45
1.25	1.07	1.25	0.59	0.70	0.90	1.13	1.08	1.49	0.92
1.17	1.16	1.26	0.24	1.10	1.99	0.87	1.51	1.21	1.27
1.10	2.35	1.01	8.82	1.85	0.63	1.06	1.65	1.02	0.66
1.03	0.42	0.86	1.09	0.87	0.61	1.23	0.20	1.00	1.55
0.66	1.51	1.90	2.62	0.90	1.65	1.13	0.59	1.06	0.86
0.78	0.76	1.23	0.98	0.85	1.29	0.99	0.77	0.94	1.18
1.17	1.32	1.41	1.12	1.07	1.68	1.00	0.62	1.39	0.77
1.09	1.48	0.84	0.57	0.91	2.27	1.09	2.04	0.98	2.38
0.97	2.41	0.90	0.55	0.68	1.40	1.41	0.77	3.18	0.12
2.92	0.72	1.14	2.50	0.93	1.25	1.12	1.04	0.98	0.40
1.15	0.67	0.95	1.16	1.91	1.04	1.25	1.25	1.00	0.22
1.02	3.34	1.02	4.08	0.68	6.87	1.16	2.11	1.16	2.30
0.84	0.30	1.71	2.55	0.66	0.42	1.03	3.71	0.99	2.68
0.85	0.55	0.97	1.30	0.85	2.16	1.04	0.55	0.90	0.80
1.16	0.73	0.90	1.38	0.99	0.14	1.09	5.24	1.09	0.58
1.32	0.54	0.86	2.30	1.21	0.31	0.92	1.08	1.11	1.04
1.52	0.15	0.66	0.15	1.10	0.62	1.19	11.47	1.08	2.13
0.97	0.99	1.09	0.90	0.88	0.68	0.66	0.71	1.18	3.78
0.90	1.16	0.81	0.94	1.38	0.16	1.16	1.07	0.88	0.36
1.43	0.59	1.87	0.74			1.14	0.58	1.03	1.06

Chapter 4

DISCUSSION

This study explored *Bdnf* DNA methylation at exons IV and IX in two tissues, PFC and blood, following exposure to aversive caregiving. After collapsing by perfusion and sex, the maltreatment group showed increases in *Bdnf* methylation at exons IV and IX in the PFC and in blood compared to normal care (Fig. 9). Additionally, cross-foster animals exhibited increased methylation relative to normal care in blood but not PFC at both exons (Fig. 9). No significant correlations were found between *Bdnf* methylation in the PFC and blood at either exon, but about half of the samples exhibited similar directional changes in methylation in both tissues.

Table 2. Summary Table of *Bdnf* DNA Methylation Results after collapsing across perfusion and sex. Data is summarized from the current study and previous PFC findings at PN8 from Roth et al., 2009 and Doherty et al., 2019.

		Exon	
		IV	IX
Tissue	Blood	↑ in maltreatment and cross-foster	↑ in maltreatment and cross-foster
	PFC	↑ in maltreatment	↑ in maltreatment
	Previous PFC findings	no change	↑ in maltreatment

4.1 Perfusions Do Not Impact Results

Regardless of sex, exon, or tissue type, there were no main effects of perfusions. The small amount of blood in the PFC of animals who were not perfused does not significantly impact results when quantifying DNA methylation. Although an unsurprising finding, this result is valuable in that it suggests that in future studies it is

not necessary to perfuse PFC tissue to obtain an accurate reading of brain DNA methylation.

4.2 Aversive Caregiving Increases *Bdnf* DNA Methylation in the PFC

The scarcity-adversity rodent model for low nesting resources demonstrated increased DNA methylation at exons IV and IX in the maltreatment group compared to normal care. In the case of exon IX this is in accordance with previous findings, but previously no change in methylation has been seen at exon IV following this paradigm at this age (Doherty et al., 2019; Roth et al., 2009). This study obtained most animals from a different vendor (Charles River) compared to previous studies published by this lab, a possible explanation for this discrepancy. Additionally, the current study had a larger sample size and smaller standard error than these previous reports. The current study's findings are, however, similar to previous findings in adult rodents at exon IV (Roth et al., 2009). Perhaps there is a small increase in infancy that becomes greater and more noticeable in older rodents.

The human literature is somewhat in agreement with the current finding. Expression of the exon IV transcript does change in the PFC over the course of normal development, with expression being lowest at birth and highest in infants and toddlers (Wong, Webster, Cassano, & Weickert, 2009). This suggests that the expression of exon IV may be susceptible to alterations in these early years of development, however; further research is needed to determine how child maltreatment may alter methylation at exon IV early in the lifespan.

4.3 Aversive Caregiving Increases *Bdnf* DNA Methylation in Blood

The maltreatment group also exhibited higher methylation in blood at exons IV and IX compared to normal maternal care. This is in accordance with the human literature, which has found increases in DNA methylation at exons I, IV, and VI in the blood of adults who experienced child maltreatment (Perroud et al., 2013; Thaler et al., 2014; Unternaehrer et al., 2015). Human research examining alterations in DNA methylation at exon IX is limited, but this exon does also seem to be susceptible to changes in DNA methylation in human blood (Hsieh et al., 2019).

Changes in *Bdnf* DNA methylation due to aversive caregiving that occur in the PFC seem to be reflected in the blood. This is in accordance with a previous study that found similar changes in DNA methylation in the hippocampus and in blood following a different model for early-life adversity (Kundakovic et al., 2015) and with research that has found correlations between DNA methylation in the frontal cortex and in blood (Horvath et al., 2012; Ursini et al., 2011). Thus, blood *Bdnf* DNA methylation may indeed be an appropriate proxy for methylation changes in the PFC in the context of aversive caregiving.

However, a noteworthy difference between the PFC and blood data is the higher variability in DNA methylation among samples within a given group in blood compared to the PFC. This is in agreement with previous findings that have reported higher variability of DNA methylation in blood compared to the cortex (Davies et al., 2012). It seems that DNA methylation in the blood tends to vary between individuals more than it does in the PFC. In addition to increased variability, DNA methylation increased and decreased to a higher degree than was seen in the PFC, suggesting that *Bdnf* DNA methylation in blood not only varies more between samples but exhibits a wider overall range.

4.4 Cross-foster Animals Also Exhibit Increased Methylation in Blood

Although the maltreatment group exhibited similar patterns of *Bdnf* methylation in the PFC and blood, the same cannot be said for the cross-foster group. Once perfusions and sexes were collapsed, animals in the cross-foster condition exhibited increased *Bdnf* methylation at both exons in blood, but not in the PFC. This lack of change in the PFC was expected according to previous studies (Doherty et al., 2019; Roth et al., 2009). However, this increase in the blood suggests something in common with the cross-foster and maltreatment groups is responsible for the change in methylation in that tissue. Perhaps increased handling or exposure to white noise or a novel environment led to the observed increase. Due to this result, we cannot unequivocally state that it is the aversive environment that led to the increase in DNA methylation in the blood of maltreated animals, because animals who did not experience an aversive environment also exhibited increased methylation. However, it is likely that the increased blood DNA methylation in the maltreatment group is caused by aversive caregiving, as previous animal and human literature has suggested such a change in both brain and blood tissue (Kundakovic et al., 2015; Perroud et al., 2013; Thaler et al., 2014; Unternaehrer et al., 2015).

This increase in methylation in cross-foster animals compared to normal care could be the result of experiencing a significantly more nurturing environment than the normal care group. However, this is unlikely, as increased DNA methylation seems to be linked to more aversive behaviors, not fewer, although this could be different in blood (Doherty et al., 2019; Roth et al., 2009). Additionally, this behavioral difference did not affect methylation in the PFC.

As the current study did not examine gene expression, it is unknown whether gene expression was impacted by this increase in DNA methylation in the blood.

Previously, our lab has found DNA methylation in the adult PFC to correspond to decreased gene expression, as is typically the case, but blood may not follow this pattern (Roth et al., 2009). It could be the case that methylation in the maltreatment group corresponds to decreased expression as it does in the brain, but the methylation in the cross-foster group corresponds to a different effect on gene expression (perhaps no change in gene expression or increased expression, as these animals experienced more nurturing care than the normal care group). However, further research is necessary to investigate these possibilities.

4.5 No Correlations Were Found Between PFC and Blood DNA Methylation

After running correlational analyses between PFC and blood for all groups that exhibited similar methylation changes, no significant correlations were discovered. This was the case for the original data and the log-transformed difference scores. It seems that although the mean DNA methylation for both PFC and blood increased in the maltreatment group compared to normal care, this did not occur, or at least not to the same degree, in both tissues for each individual animal. Only about half of the animals exhibited increases in both tissues or decreases in both tissues while the rest exhibited increases in one tissue but not the other. It seems that only one in two rodents exhibit *Bdnf* methylation changes in the same direction in both PFC and blood, at least after aversive caregiving.

Many previous reports examining DNA methylation correlations between brain and blood examined individual CpG sites (Braun et al., 2019; Ewald et al., 2014; Hannon et al., 2015; Horvath et al., 2012; Kundakovic et al., 2015; Ursini et al., 2011; Walton et al., 2016; Witzmann et al., 2012). The MSP method of quantifying DNA methylation used in the current study reveals a single methylation value for each exon,

so CpG site-by-site analysis was not possible. Since it seems that there are some CpG sites that show strong correlations between tissues, but many regions of the genome do not (Braun et al., 2019; Walton et al., 2016), it may be the case that the MSP method is concealing some CpG site-specific correlations within the *Bdnf* gene. If a few CpG sites showed very strong correlations, but most were not correlated at all, this effect would be washed out in MSP quantification. Additionally, one study only found strong similarities between the brain and blood when looking across different CpG sites in each tissue (Ewald et al., 2014). An effect like this would also be impossible to detect with the current method of methylation quantification.

4.6 Future Directions

While the current study lays the foundation for blood methylation research in our lab, there is still much to be discovered. *Bdnf* DNA methylation in the blood of adult rodents after aversive caregiving could be explored. This would reveal whether increases in methylation found in the current study remain throughout the lifespan or even whether novel methylation changes arise later in life, both of which have been found to be the case in the PFC (Roth et al., 2009) and other brain regions (Roth, Matt, Chen, & Blaze, 2014). Future studies may also perform cell sorting in order to determine how DNA methylation within individual cell populations in both PFC and blood tissue may change in response to aversive caregiving. Multiple cell types constitute both blood and brain tissue, and some of these cell types may correlate better than others. Additionally, using bisulfite sequencing PCR (BSP) rather than MSP would allow for the quantification of methylation at specific CpG sites. This would further our understanding of how *Bdnf* DNA methylation may be correlated at specific CpG sites but not others.

While research examining a correlation between methylation in blood and the brain has been mixed, correlations between average methylation in saliva and the brain appear more promising (Braun et al., 2019; Smith et al., 2015). As such, future research could also examine methylation in saliva compared to the PFC and blood following a rodent model for aversive caregiving.

4.7 Conclusion

The current study examined how *Bdnf* DNA methylation is altered in the PFC and blood after aversive caregiving. In both tissues, DNA methylation at exons IV and IX increased in the maltreatment group compared to normal care. However, in blood, the cross-foster group also exhibited an increase in methylation compared to normal care at both exons. Despite similar group trends in both tissues, no correlation was found between methylation in the PFC and blood, though about 50% of subjects in the maltreatment group showed the same directional trends in methylation across tissues. Overall, this study shows that changes in *Bdnf* DNA methylation due to aversive caregiving are similar in both the PFC and blood. Additionally, blood *Bdnf* DNA methylation may be an appropriate proxy for that of the brain when examining group, but not individual, cases of child maltreatment and perhaps the neurological disorders associated with it.

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Appendix
IACUC APPROVAL

Approval is confirmed below.

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

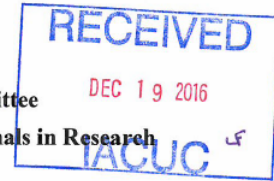
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Title of Protocol: Epigenetic mechanisms in lifelong changes in genes and behavior associated with adverse caregiving	
AUP Number: 1216-2020-0	← (4 digits only — if new, leave blank)
Principal Investigator: Tania L. Roth	
Common Name (Strain/Breed if Appropriate): Rat (Long Evans Blue Spruce) Genus Species: Rattus norvegicus	
Date of Submission: 11-18-19	

Official Use Only	
IACUC Approval Signature: 	
Date of Approval: 2.1.20	

Revised: 3/05/2019

**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	

Official Use Only
IACUC Approval Signature: <u><i>Tania L. Roth, DVM</i></u>
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