INVESTIGATION OF EARLY CHILDHOOD CARE TYPE ON METHYLATION OF BRAIN DERIVED NEUROTROPHIC FACTOR EXON IV

by

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

Spring 2022

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ACKNOWLEDGMENTS

I would like to first give my thanks to Dr. Mary Dozier, whose lab has provided me with a wonderful opportunity to learn and grow over the past two years as I explored the world of undergraduate research. I would also like to thank Christiana Martin, who has been an incredibly dedicated and helpful mentor to me, and who has taught me so not only the protocols necessary to conduct this research, but also taught me what it means to be a researcher. Both of their patience with me has been incredible throughout an arduous process to reach this final point. I would also like to thank my family and friends who saw me through my best and worst moments from start to finish, and who have provided me unwavering support through these past few years.

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ABSTRACT

Institutionalized care is a widespread form of child caregiving characterized by group care settings, high rates of caregiver turnover, and inadequate socioemotional interaction for child development. An estimated 5.37 million children are currently being raised in institutional care settings, many of which fall under the category of orphanage care. Institutionalized care has been identified as a causal factor in various negative developmental outcomes for children, such as insecure attachment, increased risk for psychiatric disorders, troubles with facial and emotional processing, accelerated maturation of neural connections, and disturbed stress responses. The mechanisms by which these negative outcomes manifest in developing children are numerous, but one of interest is DNA methylation, a dynamic form of gene expression regulation sensitive to environmental factors. Brain Derived Neurotrophic Factor (BDNF) is a protein involved in the promotion of neuroplasticity and development of neural connections during development, and whose expression is suspected to play a role in negative outcomes of early adversity associated with neural connectivity. The present study seeks to identify if differences in average percent methylation of BDNF exon IV exist across children raised in orphanage care, those who were previously institutionalized and are now living with foster families, and children living with their biological families. I hypothesized that because foster care presents a more nurturing environment compared to institutionalized care, children raised in foster care would exhibit lower levels of BDNF methylation compared to children raised in institutional care. No significant differences were identified across the three care types. These findings suggest that BDNF exon IV may not be a target for methylation in response to early adversity experienced in institutional care. Further studies should be

conducted to clarify the connection between institutional care and BDNF expression, and whether or not these changes can be identified across multiple time points.

Chapter 1

INTRODUCTION

Institutionalized care is a system of caregiving for children where children are raised in group settings. The child to caregiver ratio is high, most caregiving staff receiving training that restricts sustained contact with children, and children are more likely to receive care on a schedule rather than according to their needs. Additionally, children are unlikely to remain with a consistent group of caregivers throughout their development due to high caregiver turnover, which prevents children from experiencing the same stability experienced by children raised with biological or foster parents. This style of caregiving is incongruous with children's needs in development, with the effects being most noticeable for young children. A 2020 estimation study reviewing publications pertaining to institutionalized care statistics identified that a median estimate of 5.37 million children live in institutionalized settings globally (Desmond, et al., 2020). While regions of the world such as North and Latin America have become less favorable towards institutionalized care, it is still a normalized form of caregiving globally. Eastern European countries such as Russia and Ukraine display normalization of institutionalized care, with it even being an encouraged option for children born with visible developmental disabilities. Additionally, institutionalized care has been associated with a wide range of negative psychological and neurological effects. Given its current prevalence, it is essential to understand not only the effects institutionalized care has on children and their development, but also the mechanisms by which these effects take place. With greater understanding of

these factors, we can create informed policy changes to help affected children and improve their overall quality of life.

Time spent in institutional care, especially in the early years of development, has been shown to have negative consequences on a child's development in a number of domains, including IQ (van IJzendoorn, Luijk, et al., 2008), facial and emotional processing (McLaughlin, et al., 2014), attachment (van IJzendoorn, Palacios, et al., 2011), and risk for development of a number of psychiatric disorders including ADHD (McLaughlin, et al., 2014). Additional consequences of early life adversity have been identified in studies of brain activation. Under normal developmental conditions, children under 10-years-old display positively coupled functional connectivity between the prefrontal cortex and amygdala, and display negatively coupled functional connectivity between the two regions above age 10 (Gee, Humphreys, et al., 2013). However, in a study examining previously institutionalized children who experienced maternal deprivation from the ages of 6 to 28 months, the mature negative coupling pattern between the prefrontal cortex and amygdala was observed, indicating a developmental adaptation to early adversity via changes in neuroplasticity (Gee, Gabard-Durnam, et al., 2013). Neuroplasticity refers to the brain's ability to alter synaptic connections, oftentimes as a result of environmental stimuli. The study also concluded that affected subjects displayed disturbed reactivity of their hypothalamo-pituitary-adrenal (HPA) axis, which could indicate disrupted stress responses as a consequence of institutionalized care (Gee, Gabard-Durnam, et al., 2013). These various findings provide evidence for the notion that institutionalization, by some mechanism, results in negative physiological outcomes for the affected children.

When investigating the mechanisms by which these effects occur, epigenetics may provide some answers. Epigenetics refers to the study of how gene expression, the creation of an RNA transcript from a gene and downstream production of its encoded protein, can change in an organism's lifetime without altering the organism's genetic sequence. One epigenetic mechanism is DNA methylation, a process by which methyl groups are added to regions of DNA responsible for gene expression regulation by the enzyme DNA metyhltransferase. This typically inhibits the binding and function of transcription regulating proteins, such as transcription factors and RNA polymerase. In some cases, DNA methylation can promote transcription of a gene, such as by restricting the transcription of genes encoding transcription inhibitory proteins or impeding their ability to bind to promoters of certain genes. However, in most cases, DNA methylation directly inhibits transcription of the affected gene by preventing the binding of transcription factors to promoter regions or recruiting gene expression-repressing proteins (Moore, et al., 2012). This is a common occurrence as about 70% of gene promoters are found within 5'-Cytosine-Phosphate-Guanine-3' (CpG) islands, regions of highly conserved cytosine-guanine nucleotide linear repeats, where DNA methylation most commonly occurs (Moore, et al., 2013). Cytosine nucleotides on one DNA strand bind complementarily to guanine nucleotides on the opposite strand, and as a result, when cytosine is followed by guanine on one strand, another cytosine will be present in close proximity on the opposite strand. This is important because for methylation to effectively alter gene expression, it must occur symmetrically across both DNA strands, and therefore requires two opposite cytosine nucleotides to methylate. The high frequency of promoters being found within CpG islands makes them ideal candidates for methylation, and indicates methylation as a

gene expression mechanism more closely related to downregulation of gene transcripts.

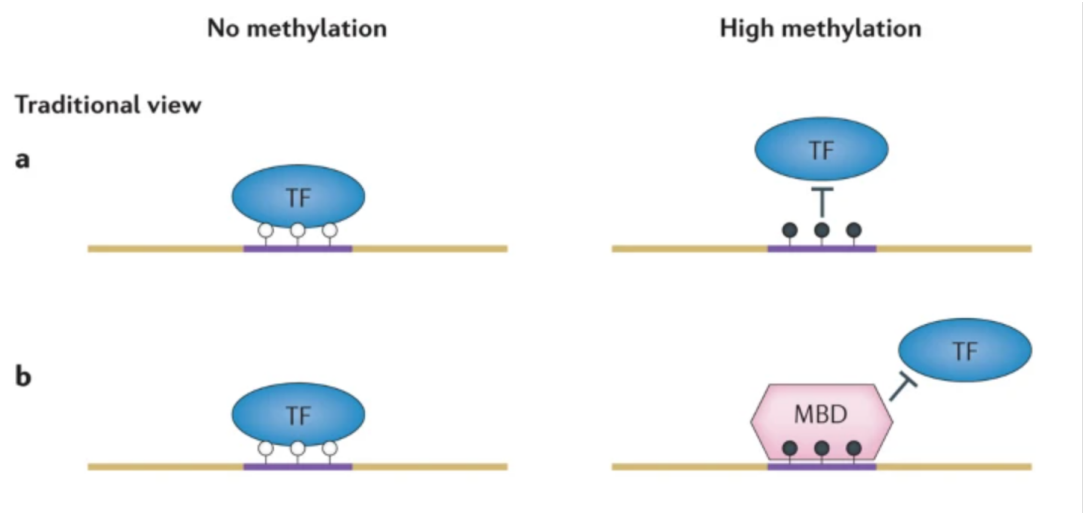

Figure 1: Display of how methylation commonly inhibits transcription through prevention of transcription factor binding. TF stands for transcription factor and MBD stands for methyl-CpG binding-domain. Figure found in Zhu et al., 2016.

Many pre-clinical studies have been conducted to examine how early adversity results in significant changes in DNA methylation. One such study examined Brain Derived Neurotrophic Factor (BDNF) methylation as well as global methylation levels in the amygdala and hippocampus of rats exposed to caregiver maltreatment within the first postnatal week. The results indicated increased methylation of the BDNF gene in females, and while no significant increase in methylation of the BDNF gene occurred for males, there was increased overall global methylation levels, suggesting that effects of maltreatment and early adversity persist into adolescence via the epigenome (Doherty, et al., 2016). BDNF is not the only

target of methylation, however, and other brain-related genes have displayed varying methylation levels in early adversity studies. One such gene is FK506 binding protein 5 (FKBP5), which encodes for a glucocorticoid receptor regulator and negative feedback inhibitor of the HPA axis, resulting in a prolonged release of cortisol in response to stress. A study examining FKBP5 methylation levels in people with major depressive disorder (MDD) observed that in individuals who carried a high risk variant of the FKBP5 allele associated with many clinical symptoms of MDD, lower levels of FKBP5 methylation were predicted by childhood adversity (Tozzi, et al., 2018). However, a different study examining individuals without significant mental health issues identified that stressful life events in childhood were associated with greater levels of FKBP5 methylation, which was found to mediate effects of early stress on prefrontal brain activity (Harms, et al., 2017). While these findings may seem inconsistent with each other, what they do indicate is that methylation levels of FKBP5 can change as a result of early adversity. Changes in either direction away from normative methylation levels are more likely to result in expression of FKBP5 capable of suppressing or heightening the stress response produced by the HPA axis. Changes in methylation in response to early adversity have been observed at various sites beyond even specific brain-related genes. One study involving parent-child dyads with Child Protective Service involvement and Attachment and Biobehavioral Catch-up (ABC) intervention examined how intervention affected DNA methylation. The ABC intervention is a parenting intervention focusing on parental sensitivity and nurturance, which has been shown to have a significant, positive effect on a child's development. The study found that children who received the ABC intervention compared to a control intervention were found to have significant DNA methylation

variation at 14,828 different sites (Hoye, et al., 2020). These studies together provide a foundation for how DNA methylation is altered both by early adversity, and also positive nurturing environments following exposure to early adversity.

One specific gene of interest in DNA methylation studies of early adversity and mental health is the BDNF gene. This gene, located on chromosome 11, is responsible for production of Brain Derived Neurotrophic Factor. Studies have shown that BDNF has significant involvement in neuronal growth and survival, modulation of neurotransmitters, neuronal plasticity, and is an important element of long-term potentiation supporting learning and memory (Bathina, & Das, 2015). The effects of decreased BDNF levels have been studied in connection with a wide number of neurodegenerative diseases and mental illnesses, including Parkinson's Disease, Schizophrenia, Alzheimer's, and multiple sclerosis (Bathina, & Das, 2015). Thus, it is essential to identify how certain environments can regulate expression of this gene to promote normative development. One study identified that adult rats that experienced maltreatment as pups displayed significant increases in BDNF methylation at exon IV and IX (Roth, et al., 2009), two exons whose expression promotes production of BDNF, with the latter coding for the protein itself. Methylation of exon IV in particular is a common focus of studies seeking to identify effects of environment on BDNF expression as dynamic methylation of exon IV has specifically been shown to regulate BDNF expression during development (Dennis & Levitt, 2005). Nine of the eleven exons in the human BDNF gene contain functional promoters and multiple alternative splicing sites exist across the gene, resulting in numerous noncoding exon targets for pre- and posttranscriptional regulation of expression, as well as various specialized forms of the BDNF protein dependent on exon expression

(Zheleznyakova, et al., 2016). Given that institutionalized care and early adversity have already been associated with changes in brain connectivity, it is possible that changes in BDNF expression could be a mechanism by which these changes occur. It is believed that methylation of this gene contributes to some of the negative outcomes experienced as a result of early adversity in infants, and it is possible that recalibration of the gene's methylation may help to ameliorate some of the associated negative consequences due to its relationship with neuroplasticity. By studying BDNF methylation in response to early adversity, more can be understood about how certain environments could potentially alter methylation, or return it to normative levels.

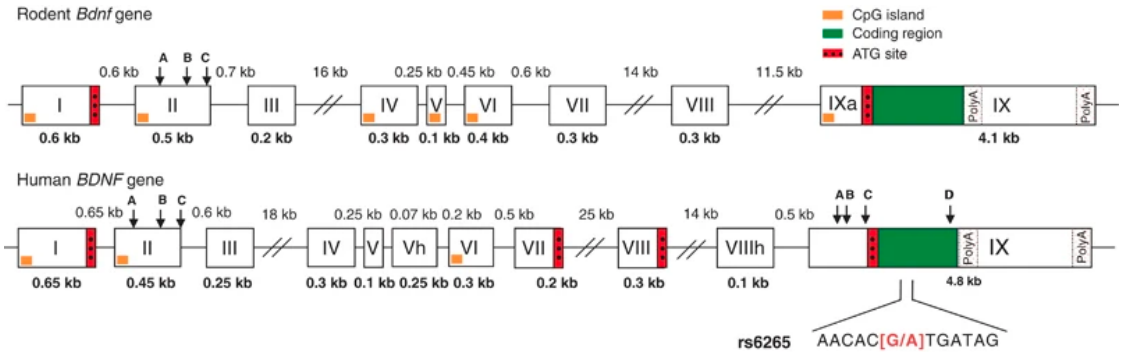

Figure 2: Differing structures of the rodent and human BDNF gene. Exons are represented by boxes and introns are represented by lines. Figure found in Boulle et al, 2012.

In order to examine this further, the current study sought to identify differences in BDNF methylation in Russian children in orphanage care and those who have been transferred from orphanage to foster care. These varying care types were also compared to a low-risk control group of Russian children raised with their biological

families, which allowed for comparisons between both the institutionalized group and the foster group to the more nurturing environment. The specific region of interest is BDNF exon IV, which has been identified to have a negative correlation between methylation levels and expression of the BDNF protein. I hypothesized that children who had been transferred to foster care would show decreased levels of BDNF exon IV methylation when compared to those still in institutionalized care, as the foster environment is likely to be more appropriately nurturing for healthy development of the children. I expected that children would adapt physiologically to a more socioemotionally nurturing environment via changes in DNA methylation.

Chapter 2

METHODS

2.1 Participants

Participants were children from the Russian Federation between the ages of 8 and 48 months. All children were considered to be typically developing, and children were excluded from the study if they had been diagnosed with any significant medical conditions that could affect normative development. Children belonged to one of three care types. The first group consisted of children who lived in orphanages (N=58). The second group consisted of children who had previously experienced orphanage care but were removed from orphanage care and placed with foster families (N=33). These children had been receiving foster care between 1 and 32 months *(M* = 15.03) prior to sample collection. The third group consisted of children who had never experienced orphanage care and had only lived with their biological families (N=33).

Of the children, approximately 55.1% were male. The average age of the children in orphanage care was 21.36 months, with their average age of institutionalization being 13.70 months. The average of the children in foster care was 25.33 months, with their average age of institutionalization being 9.00 months. The children were placed in foster care at an average age of 10.82 months, and had been in foster care for an average duration of 15.03 months. The average age of the children raised by biological families was 23.21 months (Table 1).

Between foster and biological families, most parents (68%) received at least a university education, while 20% had at least some university education, 10.7% received at least a high school degree, and 1.3% did not complete high school. As far

as family income, 17.3% reported an income above $100,000, 46.7% had an income between $61,000 and $100,000, and 36% reported an income below $60,000 annually.

No significant differences were identified across groups for any of the aforementioned demographic variables.

	Orphanage Care (N=61)	Foster Care (N=36)	Biological Family (N=39)
Gender N Males (%)	34 (55.73)	17 (47.22)	24 (61.54)
Age (months) M (SD)	21.36 (9.12)	25.33 (10.65)	23.21 (12.26)
Length of Institutional Care (months) M (SD)	7.63 (7.43)	9.76 (6.92)	---
Age of Institutionalization (months) M (SD)	13.70 (9.13)	9.00 (6.63)	---
Length of Foster Care (months) M (SD)	---	15.03 (9.07)	---
Age at Foster Placement (months) M (SD)	---	10.82 (8.64)	---

Table 1: Demographics

2.2 Procedures

Swabs were utilized to collect saliva samples from each participant by collaborators from St. Petersburg University in Russia. Samples were then stabilized using Oragene Discover-250 collection kits (DNA Genotek). Purified DNA samples were extracted from saliva with an ethanol-based method using prepIT DNA

extraction kits (DNA Genotek). Following extraction, samples were bisulfite converted via Qiagen kits, deaminating unmethylated cytosines across the genome and converting them to uracil. This process allows for identification of methylated and unmethylated cytosines following sequencing.

Samples were then divided into batches of 12, with a proportional distribution of samples from each group. The CpG rich region of BDNF exon IV (174 bp) was amplified via direct bisulfite DNA sequencing Polymerase Chain Reaction (PCR). PCR products were purified via gel electrophoresis and extracted utilizing a gel cleanup protocol (Qiagen). DNA samples were loaded into wells in an agarose gel and pushed through the gel by an electric current. Because of the porous nature of the agarose gel, DNA fragments pass through but are inhibited by their size, separating the fragments from largest to shortest the further they progress down the gel. The intended PCR product can be identified by bands on the gel visible under ultraviolet (UV) light. A section of gel containing each well's band is excised and dissolved, followed by numerous washes in order to isolate a small sample of the purified PCR product.

Purified samples were submitted to the University of Delaware DNA Sequencing & Genotyping Center for Sanger Sequencing. All samples were sequenced using reverse primer and run alongside universally methylated and unmethylated standards. Because samples were sequenced using reverse primer, sequencing results identify the complement of the template strand. Average percent methylation was calculated from sequencing results using the total number of adenines and guanines per CpG site, as adenine binds complementary to uracil, which represents the unmethylated cytosines, and guanine binds complementary to cytosine.

Thus, total guanines were divided by the total number of adenines and guanines in order to determine the percent of methylated cytosines per CpG site.

2.3 Analysis

Average percent methylation was calculated for every CpG site successfully sequenced and with legible results across BDNF exon IV of each sample and average percent methylation was derived across all CpG sites contained with the region of BDNF IV sequenced. One way ANCOVAs were conducted to determine significant differences in percent methylation between the institutional care, foster care, and biological family groups, controlling for child age and batch number.

Chapter 3

RESULTS

Data analyses were conducted utilizing Statistical Package for the Social Sciences (SPSS) software. The goal of the analysis was to identify if there were significant differences in average percent methylation of BDNF exon IV between the care type groups (M = 33.01, Range = 78.80).

A univariate analysis of covariance (ANCOVA) was run in order to identify any significant differences between groups while controlling for age at the time of sample collection and sequencing batch. Child age was included as a covariate because BDNF is a gene involved in development, and therefore age should theoretically have an effect on the regulation of its expression. Sequencing batch was also included as a covariate due to its significant positive correlation with date of sequencing (Pearson correlation coefficient r = 0.931). This allows batch number to be used instead of sequencing date given that sequencing batch effectively captures variation as a result of sequencing time. Because data assaying occurred over a period of time, factors such as slight differences in handling or reagents, lab conditions on the date of sequencing, equipment functioning and by association, batch number can account for variation in the data set.

There were no significant differences in average percent methylation between the orphanage care group (M = 33.387, SE = 2.879), foster care group (M = 33.253, SE = 3.727), and biological family care group (M = 32.161, SE = 3.765) when controlling for child age and sequencing batch [$F(2, 90) = 0.041$, $p = 0.960$, partial $\eta 2 = 0.001$]. Child age, as a covariate, did not have a significant effect on average percent methylation [$F(1, 90) = 0.318$, $p = 0.574$, partial $\eta 2 = 0.004$], while

sequencing batch did have a significant effect on average percent methylation [$F(24, 90) = 2.012$, $p = 0.010$, partial $\eta 2 = 0.349$]. This indicates that, regardless of age, care type has no significant influence on average percent methylation of the BDNF IV exon. The model was later re-run, excluding age as a covariate, and results remained consistent ($p > 0.05$). Further analysis of individual CpG sites were consistent with the primary analysis, with no significant group differences observed for each site ($p > 0.05$).

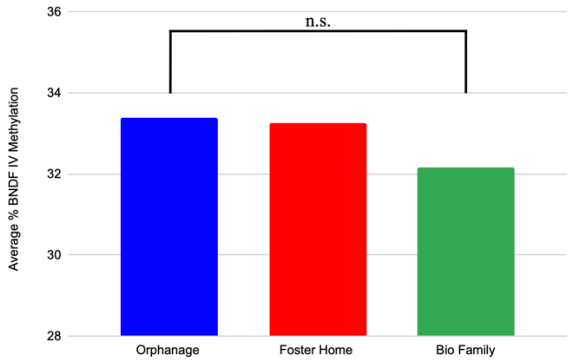

Figure 3: Mean values of average percent methylation across care types.

Chapter 4

DISCUSSION

Children from the Russian Federation in orphanage care, foster care, and biological families did not differ significantly in levels of BDNF exon IV methylation.

These null findings in average percent methylation between children raised in orphanage care and those who were transferred to foster care are consistent with findings of a previous study conducted in rodent models of cross-fostering. Roth and Sweatt (2011) found that pups of maltreated dams who were then transferred to the care of non-maltreated dams did not display a complete rescue of BDNF methylation from the increased levels displayed in pups who remained in the care of maltreated dams (Roth & Sweatt, 2011). While this is an animal model study, it is one of the closest models examining the effects of "fostering" on BDNF methylation. Numerous studies have examined positive psychological outcomes of foster care for previously institutionalized children, such as Smyke et al. (2010), which identified higher rates of secure attachment among children who were transferred into foster care compared to those who remained in institutional care (Smyke, A, et al., 2010). However, not many studies have been conducted that show BDNF methylation to mediate the effect of foster care on improved psychological outcomes. Further studies should be conducted in the future to examine if methylation, whether in different exons of BDNF or other brain-related genes, could be attributed to positive outcomes of foster care in children who were previously institutionalized.

Children in orphanage care and those who lived with their biological families did not differ significantly in average percent methylation. Countless studies have

shown deleterious effects of orphanage care in meeting children's socio-emotional needs, leading to negative developmental outcomes in areas such as IQ, risk of various psychiatric disorders, and attachment (Dozier et al., 2012; van IJzendoorn, Luijk, et al., 2008; van IJzendoorn, Palacios, et al., 2011; McLaughlin, et al., 2014). Additionally, increases in BDNF methylation have been shown to be associated with early-life adversity in both animal and human models (Roth, et al., 2009; Unternaehrer, et al., 2015; Kundakovic, et al., 2015). Extrapolating from this, knowing that orphanage care represents an adverse environment for young children, we would expect to observe a significantly higher level of BDNF methylation among children raised in institutionalized care than children who were raised with their biological families. However, this was not the case, suggesting that BDNF exon IV methylation may not be an effective biomarker for measuring physiological changes as a result of institutionalized care. Another point to consider is that while there is research suggesting genes like BDNF can be properly extracted for methylation studies from human saliva (Smith et al., 2015), most if not all findings pertaining to BDNF methylation and adversity have been from direct central nervous system tissue samples or from blood samples rather than saliva, which was used for this study. Therefore, BDNF methylation findings from previous studies may be inapplicable due to differences in methods of sample collection.

4.1 Strengths and Limitations

One strength of the experiment was the uniqueness of the groups studied. Having three groups of participants, all of whom come from the same international location, allows for not only comparisons of methylation in institutionalized children

to low risk children from biological families, but also comparisons of children who have left institutionalized care for a more nurturing foster environment. Additionally, the use of BDNF as a highly studied gene pertaining to early adversity allowed for a solid foundation from which information could be used to interpret the results.

The study also had its fair share of limitations, however. One was the lengthy amount of time required to conduct numerous protocols to reach the steps for sequencing. Sample processing from start to finish could take anywhere between three days to multiple months depending on pace. Because of this, there were numerous opportunities for error within each protocol, with the consequences sometimes being undetectable until the final quantification steps or sequencing. These opportunities for error could include contamination of samples prior to sequencing, exposure of DNA to dangerous temperatures, and degradation of sample in storage over time. Identifying where errors occurred via troubleshooting could then take significant amounts of time, which led to the significant batch effects identified in statistical analysis. Also, because there were no qualitative or quantitative measurements of caregiving quality within the groups of institutionalized, foster, and biological families. Therefore, variability in care received within the groups could not be accounted for.

4.2 Future Directions

There are a number of different directions from which this research can be expanded. While this study focused on mean differences in percent methylation across groups at one time point, there are other ways to identify effects of care type on methylation. There was a significant amount of variability across participants, independent of group, which could suggest individual factors present in different

participants that accounted for noticeable variability in percent methylation. Because of this, it might be worthwhile to examine how percent methylation of BDNF exon IV changes across two time points, rather than identifying group differences at a single time point. Considering dynamic methylation of exon IV plays a significant role in expression of BDNF in development, changes over time might be more noticeable in response to environment.

It would also be worthwhile to examine different exons beyond exon IV. Exons such as exon IX, which encodes for the protein itself, or other noncoding exons with significant effects on expression, such as exon I, might be targets for methylation as an adaptive response to environment for BDNF in institutionalized care settings, and could account for the lack of significant differences across groups observed in exon IV. In addition, while measurements of average percent methylation may be useful for purposes of comparison, they are not necessarily the most effective at measuring direct expression of gene products. Directly measuring expression of BDNF mRNA might help to elucidate some of the mysteries surrounding the effects of institutionalized and foster care on BDNF methylation.

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