CHARACTERIZING BDNF METHYLATION IN THE ADULT INSULA FOLLOWING EARLY-LIFE CAREGIVER MALTREATMENT IN RATS

by

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ABSTRACT

Adverse caregiving early in life can result in long-lasting changes in brain function and behavior and has been linked to aberrant behavioral outcomes and psychopathology later in life. Animal models have shown similar changes and outcomes. One way that changes in brain and behavior are conferred by early life adversity is through epigenetic changes such as DNA methylation. DNA methylation is an epigenetic modification that can alter gene expression without changing the sequence of nucleotide bases and is generally associated with decreased gene expression. We have previously shown that rats exposed to a maltreatment regimen repeatedly throughout their first week of life have DNA methylation changes in the Brain-derived neurotrophic factor (Bdnf) gene in adulthood in the whole prefrontal cortex, medial prefrontal cortex, amygdala, and hippocampus. Bdnf plays an important role in neural development and plasticity and is implicated in various psychiatric disorders. The aim of the current study was to assess Bdnf methylation and gene expression changes in an additional brain region, the insula. The insula is involved in cognitive, sensory (i.e. soft stroking touch), and emotional domains in humans. It has alsobeen implicated in psychiatric disorders such as depression, anxiety, and mood disorders. Using a within-litter design, pups were exposed to an adverse (maltreatment) or nurturing (cross-foster care) caregiving environment outside of the home cage for 30 minutes a day for postnatal (PN) days 1-7. Remaining pups were left in the home cage with the biological mother. Brains were removed on PN 90 and DNA methylation and gene expression were measured for Bdnf exon IV. Results

indicated that adult males nurtured early in life had less unmethylated Bdnf IV DNA, although this group had no changes in gene expression. Further, maltreated males had no changes in methylation but a significant decrease in Bdnf mRNA compared to normal controls. Although more analyses are needed to confirm methylation findings, these data suggest that the insula could be a new region of interest for stress-induced DNA methylation and/or gene expression changes.

Chapter 1

INTRODUCTION

1.1 Child Maltreatment

The national abuse statistics from 2013 reported an estimate of 679,000 victims of child abuse, with the highest rate of victimization occurring during the first year of life (National Abuse Survey, 2013). Child maltreatment can lead to an array of cognitive and behavioral deficits (Kolla et al., 2013; Forsman et al., 2013; Vares et al., 2015; Geoffroy et al; 2015; Gonzalez et al., 2016), and has been linked with many measures of poor somatic health (i.e. cardiovascular disease and cancer, (Felitti et al., 1998). Child maltreatment has also been implicated in a variety of mental health disorders. For example, maltreatment predicts perinatal mood and anxiety disorders that occur during or after pregnancy, alcohol dependence, and nicotine dependence (Choi & Sikkema, 2015; Elliot et al., 2016). Maltreatment is associated with a greater risk for developing PTSD and bipolar disorder (Degennaro et al., 2013; Kauer-Sant'Anna et al., 2007; S. Park et al., 2014; Poletti et al., 2015). Further, mood dysregulation and instability (Teicher, Ohashi, Lowen, Polcari, & Fitzmaurice, 2015) and increased aggression, violence, and rule breaking (Weder et al., 2009; González et al., 2016) are common outcomes in those who suffer from child maltreatment. The profound influence maltreatment has on mental and somatic health is clear; however, mechanisms by which these experiences produce such outcomes are still largely a mystery.

1.2 Epigenetics

The field of epigenetics investigates how the environment interacts with our genome and is providing a possible explanation for how child maltreatment could lead to the aforementioned phenotypic outcomes. Epigenetic modifications include histone acetylation and deacetylation, histone methylation and demethylation, and DNA methylation, which is one of the most widely studied forms of epigenetic marking (Handy et al., 2011). DNA methylation occurs through the addition of methyl groups (CH3) to cytosines, typically at CG dinucleotides (commonly referred to as CpG sites) and is generally associated with gene silencing (Bird, 2002; Fuks, 2005; Jones, Hamilton, & Voinnet, 1999; Razin, 1998; Turker, 2002). DNA methyltransferases (DNMT1, DNMT3A, DNMT3B) are responsible for the addition of methyl groups to CpG sites (Gavin et al., 2013). One mechanism by which DNA methylation silences gene activity is via prevention of transcription factor binding at gene promoters. Another mechanism involves methyl CpG binding proteins such as MECP2, which contain methyl-CpG binding domains that allow these proteins to bind to methylated CpG sites and attract a co-repressor containing a histone deacytelase complex which helps condense chromatin structure, thus decreasing DNA accessibility. While the majority of CpG sites are methylated in the genome, those that are not tend to occur in clusters called CpG islands that are found at important areas for transcription such as the promoter region of a gene (Bird, 1986).

Active DNA demethylation has been reported in post-mitotic cells and could even provide a mechanism to reprogram previously pathologic neurons (Gavin et al., 2013). Though less understood, DNA demethylation occurs through a variety of mechanisms, including the removal of methyl groups by MBD2, the removal of the methylated base or derivative by glycosylases followed by DNA repair, and/or

through the activity of TET proteins capable of oxidizing methyl-cytosines to hydroxymethyl-cytosines (Detich et al., 2002; Ooi et al, 2008; Dalton et al., 2012; Niehrs et al, 2012).

1.3 Early Life Stress and DNA Methylation

Previously, DNA methylation/demethylation were thought to only occur during early embryonic development and cellular differentiation processes. One of the first studies to provide evidence for the capability of the postnatal environment to influence DNA methylation was a rodent study conducted by Weaver and colleagues in 2004. This study found methylation of DNA associated with the glucocorticoid receptor (GR) gene that was directly associated with the type of caregiving received during the first week of life (Weaver et al., 2004). Specifically, investigators showed that specific sites within the exon 1 GR promoter were differentially methylated in response to high levels of licking and grooming/nursing versus low levels of licking and grooming/nursing (Weaver et al., 2004). Studies since have shown that stressful experiences, especially those during early postnatal development, can affect DNA methylation patterns for many genes that in turn influence behavior, cognition, and disease (Labonte, 2012; Meaney, 2015; St-Cyr & McGowan, 2015; Jawahar et al., 2015; Anier et al., 2014; Bohacek et al., 2015; Hing & Potash, 2014).

Studies of early life stress in humans have yielded data that confirm the effect of early life stress on the epigenome. A systematic review of reported DNA methylation changes in relation to child maltreatment and psychiatric disorders found epigenetic changes to occur most consistently at stress-related genes (e.g. HPA axis) and genes related to processes of neuroplasticity (e.g. Bdnf) (Hing, 2014). For example, child maltreatment and stressful events are associated with epigenetic

modifications of the GR gene (Radtke, 2015; van der Knaap, 2014). In addition, increased methylation of the GR promoter has been found in individuals with both a history of childhood trauma and diagnosis of Bipolar Personality Disorder (BPD, Martin-Blanco et al., 2014). In regard to neuroplasticity-related genes, a study examining the relationship between childhood trauma and Bdnf levels found that children exposed to trauma had increased plasma BDNF levels and inflammatory cytokines (Bücker et al., 2015). As the authors postulate, it is possible that the increased BDNF levels could be acting as a protective factor against the inflammatory markers. Traumatic experiences have also been associated with lower levels of BDNF in bipolar patients (Kauer-Sant'Anna et al., 2007). Other studies in humans have likewise found an association between low levels of parental care and stressful early-life environments and aberrant DNA methylation in offspring (e.g. Beach, 2016, Naumova, 2016; Smearman et al., 2016; Booij, 2015; Perroud et al., 2016).

1.4 Brain derived neurotrophic factor and stress-induced genetic alterations

Bdnf is essential for differentiation, neuronal survival, growth, and plasticity in the central nervous system (CNS). The protein is active at synapses, the site of cell communication in the brain, and could play a role in vesicle docking in important areas of synaptic plasticity such as the hippocampus (Bramham & Messaoudi, 2005; Pozzo-Miller et al., 1999). Polymorphisms in the Bdnf gene or aberrant methylation (resulting in lower BDNF protein levels) are implicated in learning and memory deficits (Caccamo, Maldonado, Bokov, Majumder, & Oddo, 2010; Egan et al., 2003; Radecki, Brown, Martinez, & Teyler, 2005) and disorders such as schizophrenia (Sahu et al., 2015; Jindal et al., 2010; Muglia et al., 2003; Neves-Pereira et al., 2005; Ray, Weickert, Wyatt, & Webster, 2011), anxiety (Chen, Yu, Liu, Zhang, & Zhang, 2015;

M.-H. Park et al., 2015), bipolar disorder (Fernandes et al., 2011; JJ Rakofsky1, KJ Ressler2, 2013; Kauer-Sant'Anna et al., 2007), depression (Kim et al., 2007; Lee, Kim, Park, & Kim, 2007; Martinowich, Manji, & Lu, 2007; Schumacher et al., 2005), PTSD (Martinotti et al., 2015; Su et al., 2015), and Alzheimer's and Parkinson's disease (Connor et al., 1997; Mogi et al., 1999; Phillips et al., 1991). Maltreatment-induced epigenetic modifications of BDNF may be one underlying implication in psychiatric disorders: Many studies report lower levels of BDNF in these (Connor et al., 1997; Hauck et al., 2009; JJ Rakofsky1, KJ Ressler2, 2013; Karege et al., 2002; Kim et al., 2007; Lee et al., 2007; Mogi et al., 1999; Phillips et al., 1991; Ray et al., 2011) and decreased Bdnf levels in children that have experienced maltreatment (Elzinga et al., 2011; Grassi-Oliveira, Stein, Lopes, Teixeira, & Bauer, 2008; Kauer-Sant'Anna et al., 2007) and a specific relationship between maltreatment in childhood and increased methylation of the Bdnf gene has been found in borderline personality disorder patients (Perroud et al., 2013). It is important to mention that one study has reported increased levels of BDNF in trauma-exposed children (Bucker et al., 2015), suggesting the possibility of protective responses to early life stress. Indeed, results from a 2015 study in humans suggest that increased BDNF serum levels in trauma-exposed patients could be a protective factor for (i.e. prevention of) PTSD development (Su et al., 2015). However, BDNF serum levels in women suffering from mental disorders are elevated compared to healthy controls (Dotta-Panichi, 2015), suggesting that the role of BDNF in mental health disorders may vary by sex or by the disorder in question. In either case, epigenetic mechanisms are a promising candidate for maltreatment-induced variations in Bdnf and the behavioral outcomes associated with these variations.

Our lab uses a rodent model of caregiver maltreatment to better understand the association between maltreatment and epigenetic alterations of Bdnf in the brain. Early work with this model showed that maltreatment creates a lasting difference in prefrontal cortex (PFC) gene expression and DNA methylation patterns in the Bdnf gene in adult rats exposed to maltreatment in infancy, as well as in offspring of maltreated-females when they are first time mothers (Roth et al., 2009). Subsequent work has identified maltreatment-induced changes in the hippocampus and amygdala as well (Doherty, Forster, & Roth, 2016; Roth, Matt, Chen, & Blaze, 2014). Such data provide empirical support of the ability of caregiver maltreatment to produce DNA methylation alterations across behaviorally-relevant brain regions known to be affected by childhood maltreatment- the prefrontal cortex, hippocampus, and amygdala.

1.5 Insula

The current project aims to characterize Bdnf gene expression and methylation in the insula, a region of the brain the lab has never examined. The insula is widely known for its role in the interoceptive system and autonomic control as well as its role in emotional states, cognition, and conscious awareness (Craig, 2008; Gasquoine, 2014; Shura, Psy, Hurley, Taber, & Ph, 2014; W. K. Simmons et al., 2013). The insular cortex is implicated in a wide range of mental disorders including drug addiction, schizophrenia, bipolar disorder, major depression, PTSD, obsessive compulsive disorder, and eating disorders (Naqvi et al., 2007;Shepard et al., 2012; Selvaraj et al., 2013; Takahashi et al., 2010; Chen et al., 2009; Sterzer et al., 2007;

Drevets, 2000; Schienle et al., 2009; Simmons et al 2011). For example, anxiety patients have shown increased right insular activation in response to anticipation (Simmons et al., 2011) and patients suffering from major depressive disorder exhibit a decreased insular response compared to healthy controls when tested for awareness of external stimuli (Wiebking et al., 2015). Resting state connectivity of the amygdala-insula has shown an inverse relationship with emotional and behavioral dysfunction and depressive symptoms: resting state amygdala-insula connectivity in behaviorally and emotionally disrupted youth is decreased compared to healthy youth (Bebko et al., 2014). In regard to addictive behaviors, the insula is a critical neural substrate in the maintenance of nicotine addiction; damage to this region has resulted in the ability to quit smoking without relapse (Naqvi et al., 2007).

In relation to this project, the insula is a brain region that has direct involvement with the maternal relationship. Elevation of oxytocin, a neuropeptide that facilitates parental caregiving, results in enhanced insular activation in response to infant crying (Riem et al., 2011). The insula is activated when mothers are shown pictures of their offspring and is involved with the recognition of a mother's offspring in humans (Noriuchi, 2008). The medial insula has also been identified as an area activated by maternal love (Bartels, 2004). This brain region is also known to contain sensory terminals involved with soft and nurturing touch (McGlone, Wessberg, & Olausson, 2014; Olausson et al., 2002). Specifically, it contains unmyelinated mechanosensitive C fiber afferents that are believed to be involved in these types of touch (McGlone et al., 2014).

As one might then expect, this region is also responsive to disruption of the caregiving relationship. In a rodent (degu or octodon degus) study of parental

separation, separated infants exhibited increased density of tyrosine hydroxylase, an enzyme essential for dopamine synthesis, in the agranular insular cortex when compared to normal controls (Poeggel et al., 2003). Trauma exposed children also show aberrant insula connectivity in the salience network, a network important for communication and social behavior (Marusak & Thomason, 2015; Menon, 2015).

1.6 Rationale

Our lab has previously reported methylation alterations as a result of early life maltreatment in the whole and medial prefrontal cortex, the amygdala, and the hippocampus (Blaze et al., 2013; Doherty et al., 2016), and the insular cortex has major connectivity with these regions (Anand, Li, Wang, Lowe, & Dzemidzic, 2009; Baur, Hänggi, Langer, & Jäncke, 2013; Cauda et al., 2011; W. K. Simmons et al., 2013) The insula region and our gene of interest are known to be sensitive to touch and caregiving (McGlone et al., 2014; Noriuchi, Kikuchi, & Senoo, 2008). They have also been implicated in many psychiatric disorders (Bebko et al., 2014; Simmons et al., 2011; Wiebking et al., 2015; Bebko et al., 2014; Naqvi, 2007). The purpose of this project is to determine whether different caregiving conditions experienced by rat pups in the first postnatal week alter Bdnf gene methylation and expression in the insula.

Chapter 2

METHODS

2.1 Subjects

Long-Evans rats were bred in our breeding colony for this experiment and were housed in polypropylene cages with generous bedding in a temperature and light controlled room (12 hours light/dark, light on at 6:00am). All animals in the colony room were given unlimited access to food and water. Postnatal day (PN) 0 signified the day of birth and on PN1 litters were culled to 6 males and 6 females. A total of 69 male and female rats from 7 litters were used for this study. No first-time mothers were used. The University of Delaware Institutional Animal Care and Use committee approved all procedures.

2.2 Caregiving Manipulations

Using a within-litter design, each experimental pup was exposed to its respective caregiving condition for 30 minutes per day from PN1 through PN7. The maltreatment condition consisted of a lactating dam placed in a novel environment with inadequate nesting material (100 ml of wood shavings). These conditions have been shown to consistently produce aversive behaviors towards infants in our lab (Blaze & Roth, 2013; Blaze, Scheuing, & Roth, 2013; Roth, Lubin, Funk, & Sweatt, 2009; Roth, Matt, Chen, & Blaze, 2014a). The cross-foster care condition consisted of a lactating dam given one hour to habituate to the experimental chamber and given

abundant nesting shavings (about 2cm evenly spread across the chamber). Remaining littermates stayed with the biological mother during the 30 minute exposures (normal care condition); these pups were only marked for identification, weighed, and immediately returned to the home cage. The temperature in the exposure chambers was kept at 27-30°C to maintain pup body temperature. All caregiving exposures were conducted during the light cycle. At the end of each caregiving session pups were returned to the biological mother in the home cage. Pups remained undisturbed until PN21-23 when they were weaned for housing in same sex pairs according to condition.

Nurturing and aversive behaviors were scored for all 3 conditions through live and video recordings of each caregiving session. Audible and ultrasonic (40 kHz) vocalizations emitted by pups during caregiving sessions were also scored. This frequency was used because it is generally emitted by pups in distress (Hofer, 1996; Portfors, 2007; Wohr & Schwarting, 2013). For caregiving behaviors, nurturing versus aversive behaviors were summed in 5-minute intervals over the 30-minute exposure time. They were then averaged across the 7 exposure days. Vocalizations were scored by marking if a vocalization was present or not for each minute in the 30-minute period and averaged across 7 exposure sessions.

2.3 DNA Methylation and Gene Expression Assays on Adult Tissue

Animals were sacrificed at PN90 via rapid decapitation under light isoflurane anesthesia. The stage of estrous was determined for females through post-mortem vaginal lavage. Brains were removed and sliced using a 1mm brain matrix. Brains were then placed on untreated slides, flash frozen with 2-methylbutane, and placed in

a -80°C freezer until later processing. The insular cortex in the rat brain was identified and dissected on dry ice using stereotaxic coordinates (The Rat Brain 6th Ed., George Paxinos and Charles Watson). DNA and RNA nucleic acids were extracted simultaneously (Qiagen AllPrep DNA/RNA kit) and quantification and assessment of nucleic acids was determined using spectrophotometry (NanoDrop 2000). Methylation status of Bdnf exon IV was assessed using methylation specific real time PCR (MSP, on Bio-Rad CFX96 systems) and direct bisulfite sequencing (BSP, on Bio-Rad CFX96 systems) on bisulfite modified DNA (Qiagen, EpiTect Bisulfite kit) as previously described (e.g. Roth, Lubin, Funk, & Sweat, 2009; Roth Zoladz, Sweatt & Diamond, 2011). For MSP, we utilized two primer sets targeting methylated and unmethylated CG dinucleotides in DNA associated with Bdnf exon IV (Blaze et al., 2013; Roth et al., 2009; Roth, Matt, Chen, & Blaze, 2014b). For BSP, bisulfite-treated samples were amplified with primer sets targeting DNA associated with Bdnf exon IV used in previous studies (Blaze et al., 2013; Lubin, Roth, & Sweatt, 2008; Roth et al., 2009). PCR products were purified (Diffinity Genomics, RapidTip) and sequenced using reverse primers at the Delaware Biotechnology Institute.

To determine gene expression levels in PN 90 tissue the extracted RNA was used for cDNA synthesis and subsequent RT-PCR reactions. Quantification and assessment of RNA quality was determined (NanoDrop 2000). Reverse transcription was performed using a cDNA synthesis kit (Qiagen) which was then amplified using real-time PCR (BioRadCFX96) with taqman probes (Applied Biosystems) to identify Bdnf exon IV or tubulin (reference gene) mRNA. All reactions for the gene of interest and reference gene in the expression assays were repeated in triplicates. The product specificity was determined through gel electrophoresis.

2.4 Statistical Analyses

Caregiver behaviors and pup vocalizations were analyzed using one- and two-way ANOVAs respectively in addition to Bonferroni's post hoc tests when needed. BSP data were analyzed with two-way ANOVAs (levels: pup condition, sex) and Bonferroni's post-hoc tests when needed. MSP data were analyzed using two-way ANOVAs (levels: pup condition, sex) and one sample t-tests (for comparisons to normal care controls). For all analyses, differences were statistically significant for $p<0.05$.

Chapter 3

RESULTS

3.1 Caregiving Manipulations

Pups were exposed to one of three conditions: normal maternal care, cross-foster care, or maltreatment. Pup-directed behaviors from the mother were recorded as either a nurturing or aversive behavior (Figure 1). Nurturing behaviors included nursing and licking of the pups. Aversive behaviors included stepping on, dragging, dropping, avoiding, and rough handling of the pups. Statistical analysis revealed a significant main effect of caregiving behavior ($F_{1,36}$) = 200, $p < .0001$) and a significant interaction between maternal behavior and infant condition ($F_{2,36}$) = 29.65, $p < .0001$). As seen in Figure 1, dams in the maltreatment condition displayed significantly more aversive behaviors ($p<.001$) and significantly less nurturing behaviors ($p<.001$) than both the normal maternal care and cross foster care dams. While dams in the maltreatment group displayed higher levels of aversive behavior than dams in the other conditions, they did display more nurturing than aversive behaviors ($p<.001$).

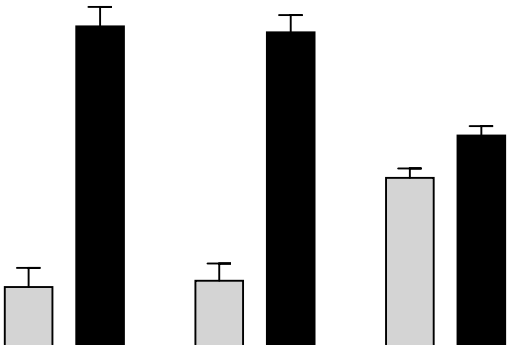

Figure 1: Overall quality of caregiving behavior amongst the three treatment conditions. Infant rodents in the maltreatment group experienced significantly higher levels of aversive behaviors and significantly lower levels of nurturing behaviors when compared to both normal care and cross foster care conditions. ***$p < .001$, ****$p < .0001$; Error bars represent SEM.

3.2 Pup Vocalizations

In addition to maternal behavior, pup responses to each caregiving condition were recorded through audible and ultrasonic (40 kHz) recordings. One-way ANOVAs and unpaired t-tests were used to detect significant differences between conditions. Audible vocalizations did not differ between treatment groups but ultrasonic vocalizations showed a significant difference among treatment groups ($F_{2,8}$=3.766, p=.0430). As seen in Figure 2, follow-up t tests for ultrasonic vocalizations showed significant differences between the maltreatment and normal care groups (p<.01) in that maltreatment pups had significantly higher vocalizations than normal care pups.

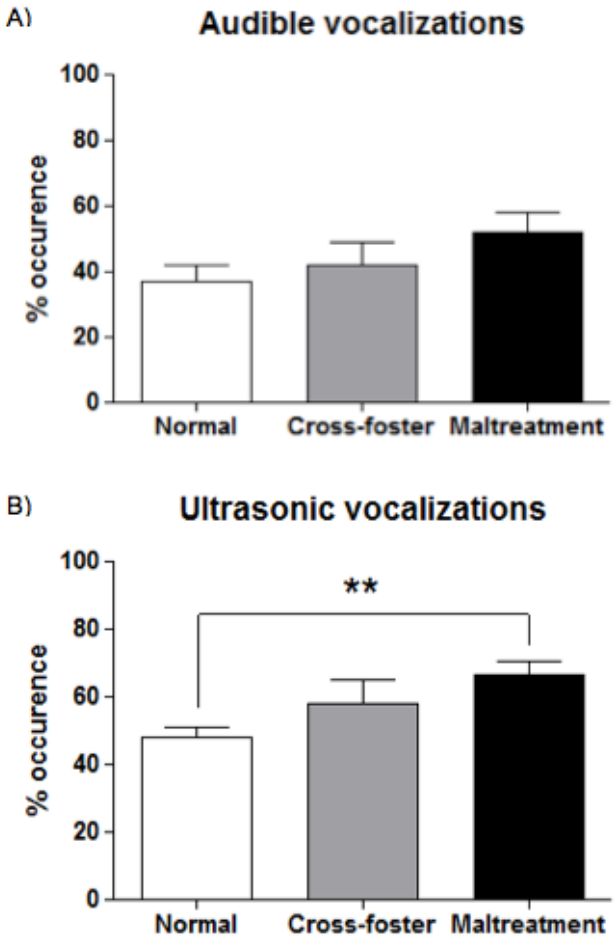

Figure 2: Pup vocalizations were recorded during each maternal care session. While the audible vocalizations (A) showed no significant differences between the three treatment groups the ultrasonic vocalizations (B) showed that the pups in the maltreatment condition vocalized significantly more than pups in the normal care condition ($p<.01$). Error bars represent SEM. N=11/12 per group.

3.3 DNA Methylation

To evaluate whether the different caregiving conditions produced changes in DNA methylation within the PN90 insula, both Methyl Specific PCR (MSP) and Bisulfite Specific PCR (BSP) were performed on all samples. Figure 3 shows the results for methylated (A) and unmethylated (B) Bdnf exon IV in adult rodents (PN

90) exposed to maltreatment or cross-foster care compared to normal maternal care animals (represented by the line at 1). A two way ANOVA revealed no interaction between methylation and treatment group and no main effect for treatment or sex in both the unmethylated and methylated assays. One-sample t tests were then completed for each group for unmethylated Bdnf IV and methylated Bdnf IV. Cross foster care males showed significantly less unmethylated Bdnf IV when compared to normal maternal care controls (T_{10} =3.659, p=.0044). Figure 4 shows the results for Bdnf methylation at 12 individual CpG sites found within the Bdnf exon IV target region. A two way ANOVA revealed a main effect of treatment in male (p<.01) and female (p<.05) Bdnf IV methylation. Two-way ANOVAs were performed for each cite and revealed no significant differences in methylation between groups (Table 1). The average methylation (i.e. when all 12 cites were averaged together) likewise revealed no significant interaction between treatment and sex ($F_{2,56}$ =0.8936, p=0.4149), and no main effect of treatment ($F_{2,56}$ =0.4661, p=.6298) or sex ($F_{1,56}$ =2.073, p=.1555).

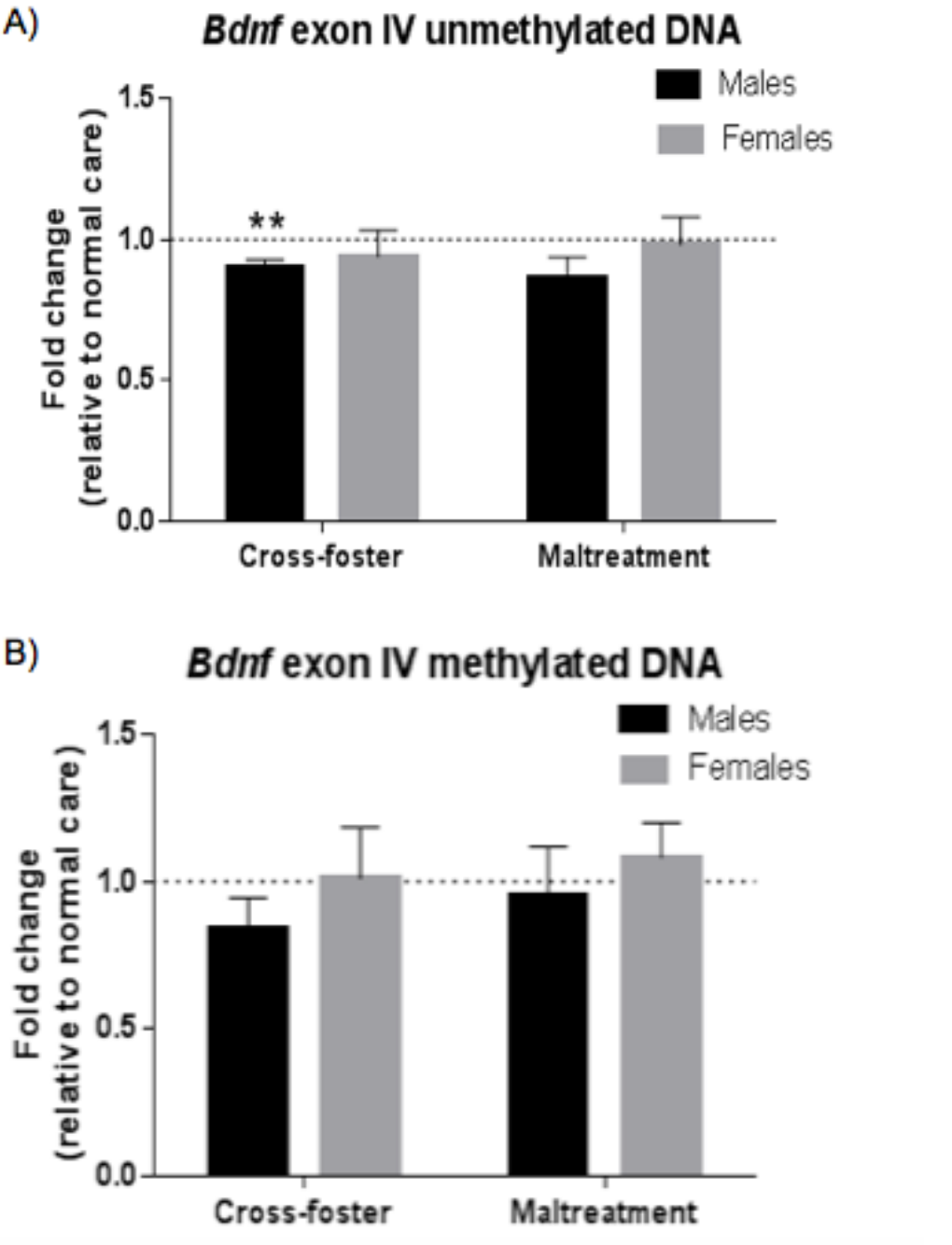

Figure 3: Methylation Specific PCR results for Bdnf IV methylation in the insula of PN90 rodents. Cross foster care males showed significantly less unmethylated Bdnf IV when compared to normal maternal care controls ($p<.01$). Error bars represent SEM. N=11-12 per group

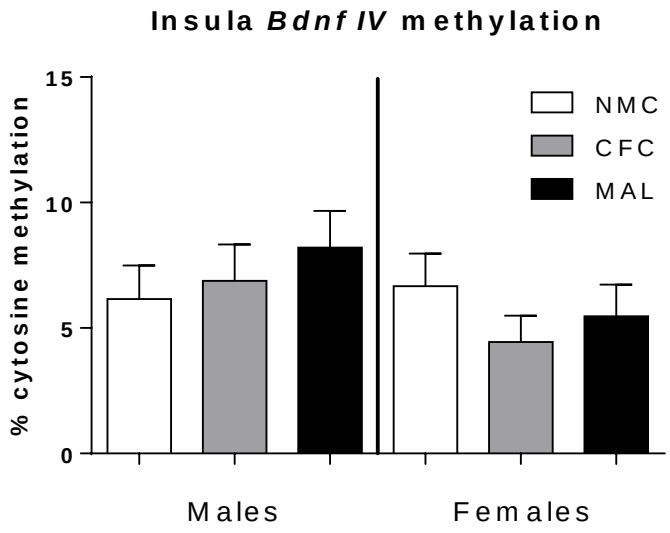

Figure 4: Bisulfite Specific PCR results for average methylation of Bdnf exon IV in the insula across 12 individual CpG sites. Error bars represent SEM. N=11/12 per group.

Table 1: Statistical analysis for individual CG site methylation.

Two-way ANOVAs for CG site methylation				
CG Site		F	d.f.	p value
1	Interaction	0.5727	2,55	0.5673
	Treatment	0.2975	2,55	0.7438
	Sex	0.3597	1,55	0.5512
2	Interaction	1.257	2,55	0.2925
	Treatment	0.2236	2,55	0.8003
	Sex	0.4965	1,55	0.484
3	Interaction	0.5917	2,55	0.5569
	Treatment	0.105	2,55	0.9005
	Sex	3.14	1,55	0.0819
4	Interaction	1.456	2,55	0.242
	Treatment	2.164	2,55	0.1245
	Sex	2.18	1,55	0.1455
5	Interaction	0.09552	2,55	0.909
	Treatment	0.6678	2,55	0.5169
	Sex	1.357	1,55	0.249
6	Interaction	0.7281	2,55	0.4874
	Treatment	1.147	2,55	0.3249
	Sex	1.666	1,55	0.02022
7	Interaction	0.1376	2,55	0.8717
	Treatment	0.2217	2,55	0.8018
	Sex	1.973	1,55	0.1658
8	Interaction	0.5861	2,55	0.5599
	Treatment	0.1786	2,55	0.8369
	Sex	2.517	1,55	0.1183
9	Interaction	0.6263	2,55	0.5383
	Treatment	0.0718	2,55	0.9308
	Sex	2.702	1,55	0.1059
10	Interaction	1.406	2,55	0.2539
	Treatment	1.556	2,55	0.2201
	Sex	1.691	1,55	0.1989
11	Interaction	0.8151	2,55	0.4479
	Treatment	0.2179	2,55	0.8049
	Sex	1.161	1,55	0.2859
12	Interaction	0.6825	2,55	0.5096
	Treatment	2.324	2,55	0.1074
	Sex	0.8233	1,55	0.3682

3.4 Gene Expression

Previous work using this maltreatment paradigm has shown significant Bdnf gene expression changes in the whole prefrontal cortex of rodents maltreated during their first week of life (Roth, Lubin et al., 2009). In the insula, in contrast, a two way ANOVA found there were no significant effects of treatment ($F_{1,41}$ =0.1924, p=0.6632) or sex ($F_{1,41}$ =0.009957, p=0.9210) and no significant interaction ($F_{1,41}$ =0.4445, p=0.5087). One sample t-tests were completed on each group and it was found that maltreated males at PN90 showed less Bdnf mRNA in comparison to normal maternal care controls (T_{10} =2.852, p<.05).

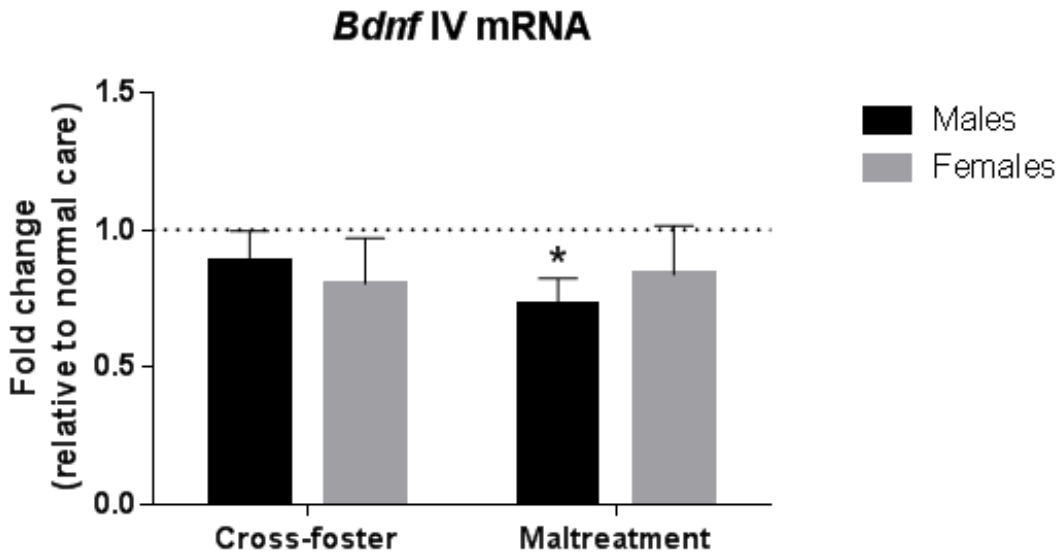

Figure 6: Bdnf mRNA levels in the adult insula. The dotted line in this figure represents the values for normal maternal care controls. Error bars represent SEM. N= 11-12 per group.

3.5 Estrous Cycle in Females

To determine if the estrus cycle (Figure 5) of adult females would have a significant effect on current methylation status, 2-way ANOVAs were performed. For gene expression (A) there was no interaction between treatment group and estrus stage ($F_{1,9}$ =0.2479, p=0.6243). The lack of differences was replicated in methylation assays (B) ($F_{2,24}$ =1.215, p=0.3144).

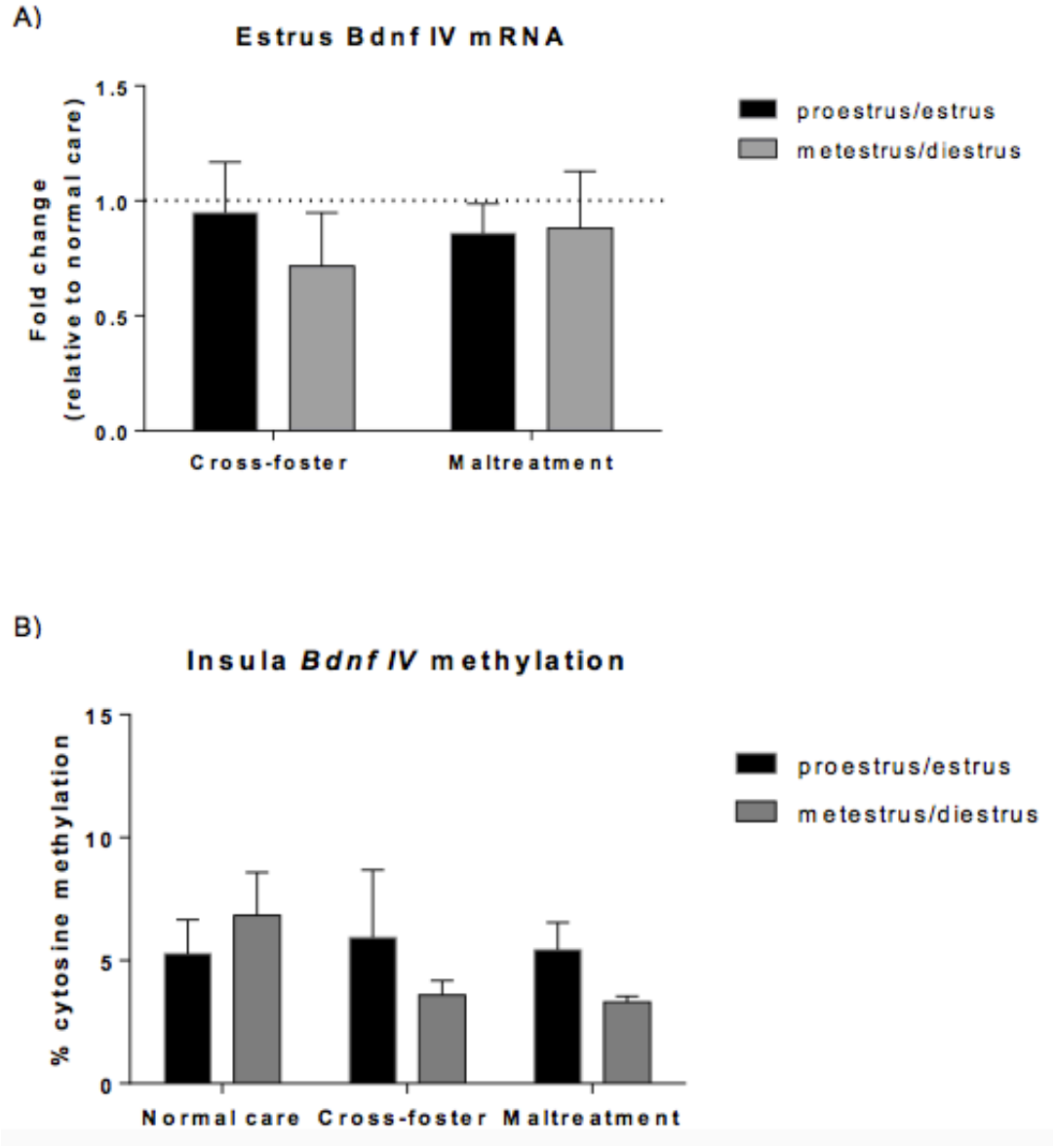

Figure 5: Comparison of gene expression (A) and methylation levels (B) of Bdnf exon IV in adult females (PN90) at various stages of the estrus cycle. To increase statistical power, the four stages were collapsed into two groups because of the similarity and temporal proximity. Figure A shows no significant differences in levels of Bdnf mRNA among treatment groups in various stages of the estrus cycle. Figure B shows no significant differences in methylation among treatment groups in various stages of estrus cycle. Error bars represent SEM. N=11-12 per group.

Chapter 4

DISCUSSION

The purpose of this study was to investigate differences in Bdnf DNA methylation and gene expression in the insula among adult rodents who experienced caregiver maltreatment during the first week of life. The results of this study support the use of animal models to study maltreatment as well as the effects maltreatment can exert on CNS DNA methylation. We were able to replicate previous results by eliciting aversive and nurturing care toward pups in our rodent model (Blaze & Roth, 2013; Blaze et al., 2013; Roth et al., 2014a). Significant differences were found in caregiving behaviors between the three conditions pups were exposed to over the first postnatal week. The normal maternal care and cross-foster care conditions both exhibited higher levels of nurturing maternal behavior toward infants whereas the maltreatment condition exhibited higher levels of aversive behaviors toward infants. Pup responses to caregiving behaviors were also found to vary among the three treatment groups. Pups in the maltreatment condition emitted significantly more ultrasonic vocalizations overall when compared to normal care controls. There were no significant differences in audible vocalizations emitted between groups. This aspect of our model provides support for the idea that pups indeed experience adversity in the maltreatment condition as infant vocalizations have been used in previous studies to quantify distress in infant rodents (Blaze & Roth, 2013; Branchi, Santucci, & Alleva, 2001; Zimmerberg et al., 2003; Marino, Cronise, Lugo, & Kelly, 2002).

4.1 Bdnf gene methylation and expression in the insula

MSP was performed to measure DNA methylation of Bdnf exon IV in the adult insula. One significant finding was that cross-foster males had significantly less unmethylated Bdnf IV when compared to normal care controls, but this was not complemented by an increase in methylated Bdnf IV, suggesting very little change in methylation. No significant changes in methylated DNA were detected. BSP was performed to verify and extend MSP data by examining methylation at each individual CpG site of the targeted Bdnf region. Statistical analyses revealed no significant differences at individual or collapsed CG sites. At the time of tissue collection all female rodents were swabbed to determine stage in the estrous cycle. There were no significant differences between proestrus/estrus females and metestrus/diestrus females in percent methylation. Thus it can be concluded that the estrus stage of females was not a significant factor altering the current methylation status of the Bdnf gene in this study.

Previously the same maltreatment paradigm was used to quantify caregiver experience effects on methylation of the Bdnf gene in other adult brain regions. In the medial prefrontal cortex Bdnf IV methylation in maltreated females was increased compared to maltreated males and female normal and cross foster care controls (Blaze et al., 2013). Maltreatment-specific differences were also found in the amygdala and the dorsal and ventral hippocampus of adult rats exposed to this paradigm in infancy (Roth 2014). In the current study we see that unlike other brain regions, the insula does not show significant differences in Bdnf IV methylation in maltreated animals. Together these data illustrate the brain region specific epigenetic effects that maltreatment is capable of producing.

A second goal of this study was to measure the level of Bdnf exon IV mRNA present in the adult insula following early-life maltreatment or nurturing care. We found that adult males that had been maltreated early in life had significantly less Bdnf mRNA (compared to normal care controls). It is possible to have gene expression changes that do not exhibit an inverse relationship to methylation changes because there are numerous mechanisms that work to drive gene expression that can occur at various stages of transcription and translation. Some examples of this include transcriptional regulator proteins that can control transcription initiation through activation or repression and post-transcriptional modifications such as RNA silencing by RNA molecules such siRNA (Xia, Mao, Paulson, & Davidson, 2002). One of the most widely studied post-transcriptional modifications occurs via short segments of non-coding RNA such as micro RNA (miRNA) that bind to complementary sequences and can recruit proteins for mRNA degradation (Bartel & Chen, 2004). Long intergenic non-coding RNA (lincRNA) have also been found to alter gene expression through guiding chromatin modifying complexes (Khalil et al., 2009).

4.2 Conclusion and Future Directions

While infant caregiving experiences early in life can have a long-term impact on gene expression and behavior, DNA methylation changes at Bdnf IV within the insula are likely not involved. Further research could help to identify which, if any, genes are experiencing expression alterations in the insula as a result of maltreatment or nurturing care in early life. Overall, this type of research will assist in mapping gene alterations resulting from early life experience as well as the locations and patterns in which they occur. Ultimately this will lead to a better understanding of how the environment can affect the epigenome and, in turn, behavior and psychopathology.

Understanding the epigenetic and phenotypic outcomes that result from maltreatment could lead to advancements in the way we treat psychopathology in patients with a history of early trauma.

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