SEX DIFFERENCES IN PUP CAREGIVING IN A
RODENT MODEL OF SCARCITY-ADVERSITY WITH MALTREATMENT

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 Bachelor of Science in Neuroscience with Distinction

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RODENT MODEL OF SCARCITY-ADVERSITY WITH MALTREATMENT

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ABSTRACT

Child abuse and neglect greatly influence the psychiatric well-being of its victims. To understand how to treat or prevent maladaptive outcomes, it is crucial to understand the biological consequences of maltreatment. Animal models are necessary to do this. Using a rodent model of caregiver maltreatment, previous research in our lab has shown sex-dependent differences in adult phenotypic outcomes and in brain methylation levels of DNA associated with the brain derived neurotrophic factor (Bdnf) gene. The reasons underlying this sex disparity are unknown, and in the current experiment, we aimed to discern whether there is a difference in the quality of maternal care received by male and female pups in our rodent model. Newborn rodents were assigned to three different experimental groups for the first seven days of life. The groups included normal maternal care from their biological dam, cross-foster care from another dam, and maltreatment from another dam. Data show that a higher percentage of adverse caregiving behaviors are directed towards female pups in the maltreatment condition. We also examined whether there were sex differences in methylation of Bdnf IX DNA in the prefrontal cortex of pups after the maltreatment. No differences in methylation were found at the infant age examined. The differences in caregiving suggest a reason for why females in this model experience greater behavioral and epigenetic consequences than males.
Chapter 1
INTRODUCTION

1.1 Childhood maltreatment as a form of early-life adversity

Experiences and environments, especially in infancy and adolescence, have a profound impact on shaping an individual’s developmental path (Holmbeck & Updegrove, 1995; Newton & Levine, 1968). Critical behaviors affected by early-life events include attachment-based learning, emotion regulation, and social behavior (Fries, et al., 2005; Loman & Gunnar, 2010; Pollak, 2008; Sullivan & Holman, 2009). Overall, the parental care a child receives during early development greatly influences the production of adaptive versus maladaptive behaviors (Francis & Meaney, 1999; Meaney 2001).

Maltreatment has been divided into four main categories: physical abuse, sexual abuse, neglect, and emotional abuse (Cicchetti & Toth, 2005). Early-life adversity, such as childhood maltreatment, has detrimental long-lasting and even transgenerational effects (Maccari et al., 2014; Pembrey, et al., 2014). Previous research has associated high reports of childhood maltreatment with an increased risk for developing depression, anxiety, abnormal aggression, and other mood disorders later in life (Cicchetti & Toth, 2005; Lee & Hoaken, 2007; Manly et al., 2001). Victims of childhood maltreatment are also more likely to have an adult criminal record when compared to individuals with no childhood maltreatment (Dutton & Hart 1992; Trauffer & Widom 2017; Widom, 1989).
1.2 Early-life adversity in rodent models

Long-term consequences of experiencing early adversity are also clear from basic research employing various animal models. Using animal models to demonstrate adversity allows researchers to manipulate early-life conditions in a reproducible manner. The current study utilizes an experimental design in which deprivation of nesting resources and inadequate habituation time is used to promote aberrant caregiving behaviors in Long Evans rats. This paradigm induces dams to display aversive behaviors (such as rough-handling, stepping on, and actively avoiding pups) and decreased nurturing behaviors (such as nursing, hovering, and licking) towards pups (Ivy et al., 2008; Roth & Sullivan, 2005; Roth et al., 2009). The aberrant caregiving produces adult behavioral outcomes that include deficits in fear extinction, increased depressive- and anxiety-like behaviors, and disruptions in maternal caregiving behavior (Blaze et al., 2013; Doherty et al., 2017; Roth & Sweatt, 2011; Roth et al., 2009).

1.3 Epigenetic modifications

Epigenetic modifications are changes to the expression of genes without the alteration of the underlying DNA sequence (Comb & Goodman, 1990). Epigenetic alterations have the ability to influence the gene expression far beyond when the actual experience occurs (Onishchenko et al., 2008; Roth et al., 2009). Because of these lasting effects, the study of epigenetics has become imperative to the fields of psychological and brain sciences to understand gene-environment interactions and their influence on neural mechanisms and behavior (Lester et al., 2011; Mann & Currier 2010).
1.3.1 DNA methylation as an epigenetic modification

Although many epigenetic modifications exist, DNA methylation is one of the most widely studied alterations in regards to behavior, due its often stable and enduring effects on gene expression (Bird, 2002). DNA methylation occurs when a methyl group is transferred by a DNA methyltransferase (DNMT) to the 5’-position of a cytosine nucleotide, which produces 5-methylcytosine (Moore et al., 2013). Often, this methylation takes place where a cytosine precedes a guanine nucleotide. Areas of the genome lush with these cytosine guanine dinucleotides (CpG sites) are referred to as CpG islands and are commonly found in gene promoter regions. Methylation of CpG islands are frequently associated with the suppression of transcription and therefore, inhibition of gene expression (Bird, 2002; Ng & Bird 1999). This inhibition is caused by one or more of the following mechanisms: blockage of transcription machinery by methyl groups, disruption of transcription factor binding to the promoter region, or attraction of co-repressor molecules such as histone deacetylases (HDACs) (Bird, 2002; Inamdar et al., 1991; Klose & Bird, 2006).

Once thought to be a fixed and immutable change to the epigenome occurring in embryonic development, we now know that DNA methylation can be altered by experiences throughout a lifetime (Blaze et al., 2013; Szyf & Bick, 2013). Though DNA methylation can be dynamic through the lifespan, alterations accumulated early on in response to environments or experiences often persist and are transmitted across generations (Champagne, 2010; Roth et al., 2009). These accumulated alterations may be responsible for behavioral outcomes. Altered DNA methylation, either genome-wide changes or at candidate gene loci, has been associated with exposure to early adversity and the diagnosis of psychopathology (Essex et al., 2013; Grayson & Guidotti, 2013; Sugawara et al., 2018).
1.4 Brain-derived neurotrophic factor gene

One gene that is greatly affected by early adversity is the brain-derived neurotrophic factor (Bdnf) gene. As a neurotrophic protein-coding gene, Bdnf is vital to neurogenesis, neuron growth, and neuron plasticity due to experience (Bath et al., 2012; Bruel-Jungerman et al., 2006; Rossi et al., 2006). It also plays an important role in long-term potentiation throughout the brain (Akaneya et al., 1997; Bekinschtein et al., 2008; Korte et al., 1995), a fundamental process for learning and memory throughout an individual’s lifespan (Jones et al., 2007; Maren, 1999). A compelling characteristic of Bdnf is that it is highly sensitive to environmental influences, meaning that experience plays a large role in expression of the gene (Klintsova et al., 2004; Tsankova et al., 2006). Changes in typical Bdnf expression result in abnormal behaviors, including psychopathology (Keller et al., 2010; Kernie et al., 2000; Lyons et al., 1999; Takahashi et al., 2000).

Research shows that highly stressful experiences in life suppresses transcription, and therefore expression, of Bdnf via increased DNA methylation (Ikegame et al., 2013; Kundakovic et al., 2013; Roth et al., 2011). Research in our lab has previously shown that repeated exposure to brief and controlled bouts of caregiver maltreatment disrupts normal expression of the Bdnf gene (Blaze et al., 2013; Doherty et al., 2016; Roth et al., 2009). These studies report that in general, maltreatment leads to increased methylation and decreased expression of the Bdnf gene (Roth et al., 2009; Blaze et al., 2013), though directional (increased or decreased) effects on methylation and gene expression are dependent on sex and brain region.
1.5 Rationale for current research

Previous research in our lab has revealed multiple sex differences in subjects after exposure to caregiver maltreatment in both adult phenotypic and epigenetic outcomes (Blaze & Roth 2013; Doherty, et al., 2017; Roth, et al., 2014). For example, male rats show deficits in fear extinction, while females exhibit changes in novel object recognition and forced-swim behavior (Doherty, et al., 2017). Following exposure to maltreatment, animals of both sexes exhibit increased DNA methylation of \textit{Bdnf} in whole prefrontal cortex as well as altered levels of various epigenetic regulators in the medial prefrontal cortex (mPFC) (Blaze & Roth, 2013; Roth, et al., 2009). In the dorsal hippocampus, males but not females have reduced \textit{Bdnf} methylation, while both males and females showed reduced \textit{Bdnf} methylation in the amygdala (Roth, et al., 2014). Female, but not male, rats subjected to maltreatment show increased DNA methylation of the \textit{Bdnf} gene that is concomitant with reduced histone 3 lysine 9/14 acetylation at the \textit{Bdnf} exon IV promoter in the mPFC (Blaze, Asok, & Roth, 2015; Blaze & Roth, 2017). Further, while maltreatment-induced methylation within the PFC has been successfully rescued using a particular drug dose and schedule in male infant rats, this same dose and drug treatment regimen was not successful in rescuing maltreatment-induced methylation in female infants (Doherty, et al., submitted).

Because we have observed sex differences in brain and behavioral outcomes in our model of caregiver maltreatment and because dams are known to differentially treat male and female offspring in the nest (Moore, 1985- dams display more nurturing behaviors towards males), this raises the possibility that during our experimental manipulations males and females are experiencing different care. In the current study, we seek to address whether there are differences in adverse care received between the
sexes. We also seek to address whether there are differences in methylation of *Bdnf* IX DNA within the PFC between male and females early in development.
Chapter 2

METHODS

2.1 Subjects

Long-Evans rats were utilized in this study and were bred in the laboratory. Multiparous Long-Evans rats were used because of their higher observed percentage of nurturing behaviors when compared to other rodent strains (McIver & Jeffrey, 1967). Dams and pups were housed in a temperature- and light-controlled (12-hour light/dark cycle) colony room in polypropylene cages (18”x9”x8”) with ample bedding and access to food and water ad libitum. Postnatal day 0 (PN0) was considered the day of birth. On PN1, litters were culled to 12 pups, keeping an even split of 6 males to 6 females when possible. A total of 60 pups from 10 experimental litters were used in this study. Procedures were approved by the University of Delaware Institutional Animal Care and Use Committee before experimentation.

2.2 Caregiving manipulations

The previously established scarcity-adversity model of rodent caregiving manipulations was used in this study (Blaze & Roth, 2013; Doherty, et al., 2017; Roth, et al., 2009). In this within litter design, experimental rats were exposed to one of three conditions: normal care in the home cage with the biological dam, cross-foster care in a novel environment from another dam, or maltreatment in a novel environment from another dam. Dams used in the cross-foster care condition received ample nesting resources (approximately 2 cm of bedding) and at least one hour of habituation time to the novel environment prior to receiving experimental pups. This environment and habituation time promoted nurturing behaviors, such as nursing or hovering over the pups. Dams used in the maltreatment condition were given low nesting resources
(approximately 100mL of bedding) and no more than 5 minutes of time in the novel environment prior to receiving experimental pups, thus causing the dam to become stressed and exhibit adverse behaviors including stepping on, dragging, avoiding, dropping, or rough handling the pups. Both the maltreatment and cross-foster manipulations were carried out in a dark room with white noise to avoid exposing dams to distractions. Stimulus dams were matched to the biological dam of the experimental pups in postpartum age and diet.

Experimental pups were chosen at random on PN1 so that only 1 female and 1 male were assigned to each of the three experimental conditions. Each experimental condition consisted of 2 pups total. To distinguish between the sexes, either all the males or all female experimental pups from the litter were marked along the back, stomach, and legs with a nontoxic marker. The sex chosen to be colored was counterbalanced across litters (i.e. females were marked in ½ of the litters and males were marked in ½ of the litters to avoid any differences in pup marking as a confound in any condition or sex differences observed). Pups were exposed to experimental conditions for 30 minutes per day for the first week of life (PN1-PN7). Pups in the normal care conditioned were marked, weighed, and returned to the home cage with the biological dam. Any non-experimental pups from stimulus dams or the experimental litter were placed on a heating pad for the 30-minute duration of the experiment. All experimental and non-experimental pups were returned to the home cage after completion of the 30-minute daily manipulations.
2.3 Behavior observations

Behaviors in all three conditions were recorded using SONY video cameras. Infant-caregiver interactions in the maltreatment condition were both live scored and recorded with SONY cameras for later coding by trained graduate students or undergraduate research assistants. For accuracy and consistency, behaviors were scored by two trained scorers and the amount of behaviors were averaged. Percent of inter-rate reliability is around 90% (Doherty, et al., 2017). Nurturing behaviors, (licking, grooming, nursing,) and adverse behaviors (stepping on, dragging, avoiding, dropping, rough handling) were recorded in 5-minute time intervals. Total adverse behaviors were also tallied throughout the entire 30-minute manipulation for each condition. Scorers were unaware of the sex of each pup and relied on the markings after coding to distinguish between sexes. After scoring, behaviors were totaled and compared between the sexes in each experimental condition.

2.4 Biochemical assays

Infant rats were sacrificed on PN8 (24 hours after the last caregiving manipulation) using rapid decapitation. The PFC was immediately isolated and homogenized and stored at -80°C until further processing. DNA was extracted using an AllPrep DNA/RNA kit (Qiagen). Quality of DNA was tested using spectrophotometry (NanoDrop 2000). Purified DNA from the PFC samples underwent bisulfite processing (Qiagen) which converts unmethylated cytosines to uracil, leaving methylated cytosines unaffected. Samples then underwent direct bisulfite sequencing (BSP) as previously described to measure DNA methylation levels (Parrish, et al 2012; Roth, et al., 2014). Bisulfite sequencing allows for the estimation of methylation at individual cytosine sites (Parrish, et al., 2012). Bisulfite-treated samples were
amplified by primer sets targeting *Bdnf* IX as used in our previous study (Roth, et al., 2009). BSP samples were sent to University of Delaware DNA Sequencing and Genotyping Center and sequenced using reverse primers. Average methylation levels across the targeted *Bdnf* region was determined using Chromas software as previously described (Parrish, et al., 2012).

### 2.5 Statistical analysis

Two-way ANOVAs followed by Tukey multiple comparison tests were used to compare differences in caregiving received (nurturing vs adverse care) and methylation between the three experimental conditions (normal care, cross-foster care, and maltreatment). T-tests were used to compare adverse behaviors received by the sexes in each experimental condition. Significance was set at p < 0.05 for all analyses. GraphPad Prism software was used to aid in statistical analyses and creation of figures.
3.1 Caregiver behavior across conditions

Two-way ANOVAs conducted on caregiver behavior across the three experimental conditions showed an interaction between caregiving behavior and infant condition ($F_{(2,54)} = 84.28, p < .0001$) and a main effect of caregiver behavior ($F_{(1,54)} = 41.83, p < .0001$) (Figure 1). Post-hoc comparisons confirmed that pups in the maltreatment condition were exposed to more adverse behaviors than pups in the cross-foster (p<.0001) and normal care (p<.0001) conditions. Further, fewer nurturing behaviors were observed in the maltreatment condition as compared to the cross-foster (p < .0001) and normal care (p < .0001) conditions. Significantly more adverse compared to nurturing behaviors were performed by dams in the maltreatment condition (p < .0001). There were no significant differences in nurturing (p = .164) or adverse (p = .3822) behaviors received by pups between the cross-foster and normal care conditions. These findings are consistent with other results using this same scarcity-adversity model and show that pups placed in the maltreatment condition experience more adverse caregiving than pups in either the cross-foster or normal care control conditions (Blaze & Roth, 2017; Doherty, et al., 2017; Roth, et al., 2009).
Figure 1. Results of caregiver behavior across conditions are comparable to previous reports. Pups in the maltreatment condition received significantly more adverse care and significantly less nurturing care when compared to normal and cross-foster care conditions. n= 9-10 pups/group; subjects derived from 10 litters; *** denotes p< 0.001. Error bars represent SEM.

3.2 Differences in caregiving received between the sexes

T-tests showed a significant difference in adverse behaviors received by the sexes in the maltreatment condition, with female pups receiving more adverse care than males ($t_{(18)} = 4.454, p = .0003$) (Figure 2a). No significant differences in aversive caregiving received between the sexes were found in either the cross-foster ($t_{(14)} = 0.4337, p = .6711$) or normal care ($t_{(16)} = 1.486, p = .1568$) conditions (Figures 2b and 2c respectively). These results demonstrate that female pups experience a greater amount of adverse care than male pups solely in the maltreatment condition.
A

**Normal Maternal Care**

![Chart showing % Adverse Behavior by gender for Normal Maternal Care]

B

**Cross-foster Care**

![Chart showing % Adverse Behavior by gender for Cross-foster Care]

C

**Maltreatment**

![Chart showing % Adverse Behavior by gender for Maltreatment with significant difference indicated]
Figure 2. No significant differences in adverse behaviors received between the sexes were found in the normal maternal care (A) or cross-foster care conditions (B). Females received a higher percentage of adverse behaviors from the caregiver in the maltreatment condition when compared to their male littermates (C). n= 9-10 pups/group; subjects derived from 10 litters; *** denotes p< 0.001. Error bars represent SEM.

3.3 DNA methylation

DNA methylation levels were measured for Bdnf exon IX in the prefrontal cortices of PN8 rats (Figure 3). A two-way ANOVA revealed no methylation difference across any of the three infant conditions ($F_{(2,48)} = 0.03, p = 0.97$) or between the sexes ($F_{(1,48)} = 0.38, p = 0.54$). Further, there was no interaction effect between sex and infant condition on methylation levels ($F_{(2,48)} = 0.48, p = 0.62$).

Figure 3. Average methylation levels of Bdnf exon IX in the prefrontal cortices of infant (PN8) rats were found using methylated primers. No significant differences were found in DNA methylation across conditions or between sexes. n= 8-9/group; subjects derived from 9 litters. Error bars represent SEM.
Chapter 4
DISCUSSION

4.1 Female pups receive more adverse behaviors than males

The first aim of this study was to explore a possible reason for why female rodents used in the scarcity-adversity model of caregiving exhibit greater behavioral and epigenetic responses to maltreatment than males used in the same model. To meet this goal, female and male pups were exposed to either a maltreatment, cross-foster, or normal care condition for the first week of life. We replicated results from previous studies which show that differences in maternal care across the three conditions used in this model. Pups in the maltreatment condition experience greater amounts of adverse care when compared to the cross-foster or normal care conditions. Further, novel data resulting from this study shows that dams direct more adverse behaviors towards female pups in the maltreatment condition when compared to male pups. We were able to analyze adverse care on a sex specific basis, but not nurturing care on a sex specific basis due to the set-up of our experimental chambers.

There are many possibilities for why female pups experience greater adverse behaviors in the maltreatment condition of our scarcity-adversity model. Male pups often experience an increase of anogenital licking from the dam because odor and hormones play a large role in the type of care exhibited by dams pups (Moore 1981; Moore, 1982). This means that dams generally direct more nurturing behaviors toward male pups (Moore & Morelli 1979). Furthermore, female rodents tend to exhibit greater locomotor activity than males (Rosenfeld, 2017). This would allow the female pups to have more frequent contact with the dam and, therefore, receive a greater amount of adverse behaviors. The design of our experimental chambers however
prevented us from being able to reliably quantify pup behaviors in this study, or quantify how much anogenital licking was directed towards male versus female pups.

Sex-specific differences in caregiving are also present in humans. Mothers provide more affectionate touching to sons over daughters (Fausto-Sterling et al., 2015) and more negative feedback towards daughters while observing them complete a task (Alessandri & Lewis, 1996). Consistent with results of studies using rodent models in the Roth Lab, human mothers who experience childhood maltreatment are more likely to exhibit aberrant care toward their children, especially female offspring (Cross, et al., 2016; DiLillo & Damashek, 2003).

### 4.2 DNA methylation of Bdnf exon IX

In this study, we also aimed to evaluate any sex differences in methylation levels of Bdnf IX in the PFC of PN8 pups. Although previous research has shown changes in methylation of Bdnf IX at PN8 due to maltreatment (Doherty, et al., 2016; Doherty, et al., submitted), we did not find any significant differences in methylation levels across conditions or between the sexes in the current study. One explanation of the lack of epigenetic differences could be because of the different methods used during biochemical analyses. Previous studies that showed sex- or condition- specific differences in Bdnf IX in infants used methylation specific real-time PCR (MSP) while this study used direct bisulfite DNA sequencing PCR (BSP). MSP is more sensitive to changes in methylation levels (Hernandez, et al., 2013; Jiang, et al., 2010; Li & Tollefsbol, 2011; Parrish, et al., 2012) and thus the technique used in the current study was possibly not sensitive enough to detect the small changes in methylation that were seen in previous studies. Future work could re-run the same DNA samples with MSP,
and examine whether there are cite-specific changes in methylation within the BSP data (instead of just looking at average methylation across the region, as done here).

4.3 Conclusion

Data lend support to the notion that one reason females in our model incur more behavioral and epigenetic consequences is a result of greater mistreatment by the dam. Future work is necessary to establish the consequences and functional implications of this sex-specific direction of adverse caregiving. This study nevertheless highlights the importance of examining the contribution of maternal care received when interpreting sex-specific outcomes.
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