PREVENTION OF ABERRANT DNA METHYLATION
INDUCED BY CAREGIVER MALTREATMENT IN EARLY LIFE

by

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ABSTRACT

Parental care is a critical buffer for normative mammalian development. While nurturing care can protect offspring from environmental stressors, poor care can have dire consequences for offspring physical and mental health. Research over the last few decades has begun to illuminate biological mechanisms involved in the influence of parental care on development, with epigenetics at its forefront. One type of epigenetic alteration that alters gene expression, DNA methylation, often increases as a result of experiencing maltreatment in early life. DNA methylation occurs when DNA methyltransferases (DNMTs) attach methyl groups to DNA, typically rendering it less accessible for transcription. DNA methylation is also causally related to behavior, making it a prime candidate for pharmacological intervention of the effects of maltreatment. The goals of this thesis were to: 1) establish an effective dose of 5-aza-2’-deoxycytidine (5-aza) that normalizes locus-specific DNA methylation in infant rats exposed to brief and repeated bouts of maltreatment; and, 2) measure the effects of the drug intervention on global DNA methylation, DNMT gene expression, and the stress hormone corticosterone. Chapter 1 reviews evidence of the influence of the epigenome in mediating the effects of early environmental influences on the brain and behavior. A major underlying theme of the chapter is the importance of animal models in establishing relationships between epigenetic mechanisms and behavior and the translational relevance of important discoveries in the field of behavioral epigenetics. Chapter 2 consists of the experimental findings of using 5-aza to prevent aberrant
methylation and gene outcomes of maltreatment. This study established an effective
dose of 5-aza that successfully lowered methylation levels in Long-Evans rat pups
when administered daily concurrent with maltreatment. Gene expression of DNMTs,
global methylation, and corticosterone concentration were measured to shed light on
the effects of 5-aza on the developing pups. Results indicate that 5-aza elicited a
condition- and sex-specific effect on corticosterone concentration, but no effect on
DNMT expression or global methylation. From this work, we have provided
additional evidence showing that even brief bouts of early life maltreatment have an
impact on cortical DNA methylation. We also show that pharmacological substances
can be effective at preventing aberrant changes in DNA methylation induced by
maltreatment. However, this project has also revealed some unexpected consequences
of the pharmacological agent later in development. Future projects in the lab will
investigate whether other pharmacological substances can prevent aberrant DNA
methylation induced by early life maltreatment, how they affect other biological
measures, and their long-term consequences for behavior.
PREFACE

I, Natalia Phillips, hold principle author status for the chapters comprising this thesis. Chapter 1 is co-authored by Dr. Tania Roth, whose contribution greatly facilitated my research. The manuscript was published in Genes in January 2019.
Abstract
The use of non-human animals in research is a longstanding practice to help us understand and improve human biology and health. Animal models allow researchers, for example, to carefully manipulate environmental factors in order to understand how they contribute to development, behavior, and health. In the field of behavioral epigenetics such approaches have contributed novel findings of how the environment physically interacts with our genes, leading to changes in behavior and health. This review highlights some of this research, focused on prenatal immune challenges, environmental toxicants, diet, and early-life stress. In conjunction, we also discuss why animal models were integral to these discoveries and the translational relevance of these discoveries.

Introduction
For more than 2000 years, humans have used animal models (e.g., dogs, chicks, pigs, rats, monkeys) to understand our own biology, behavior, and health (Ericsson, Crim, and Franklin, 2013). Animal models are incredibly important because they allow researchers to elucidate complex biological mechanisms that underlie
development, behavior, and health (Figure 1). This is something that cannot be done in human research because elucidating such requires carefully controlled experimental manipulations, such as genetic knock-out/knock-ins, hormone alterations, administration of pharmacological agents, or exposure to controlled environmental toxicants or viruses, and then the collection of brain tissue for biochemical analyses. Furthermore, animal models help control environmental variability that exists in epidemiological and clinical research (Bennett, 2007) and afford the opportunity of carefully controlled behavioral observations and measurements. In research involving human participants, it is difficult and sometimes impossible to disentangle the contribution of different factors in an individual’s life to physiological, behavioral, and health outcomes. On the other hand, animal models allow researchers exquisite control of all the factors that the animal is exposed to and to test the effects of factors individually or together (Bennett, 2007). Parsing apart the downstream consequences of a specific factor or a group of factors as well as correlational versus causative impact is critical to continue the forward momentum in developing precision medicine and treatments (Lloyd, Robinson, and MacRae, 2016).
The relationship between animal models and human studies is reciprocal: both provide important insight into biology and behavior, guide the direction of one another’s research, and complement each other’s findings. With non-human animal models, however, we can utilize genetic, epigenetic, and pharmacological manipulations to help elucidate mechanisms and establish causality between environmental factors and behavior, health, and disease outcomes.

Numerous studies have illustrated the importance of both genetic and environmental factors on biology, behavior, and health, and early evidence emerged suggesting that genes and the environment somehow interact to produce various behavioral and disorder or disease phenomena (Bennett et al., 2002; Caspi et al., 2003). We now understand this interaction to occur via epigenetics, a process by which environmental experiences induce changes in gene activity without modifying the underlying DNA sequence (Doherty and Roth, 2016; Pogribny et al., 2008).
Epigenetic alterations lead to changes in biological functions within the organism, and subsequently, changes in behavior or health (Dolinoy, Huang, and Jirtle, 2007; Tollenaar et al., 2018). These modifications can occur at any point in an organism’s life, although early development appears more vulnerable, and can even be heritable (Doherty and Roth, 2016; Parker et al., 2006).

This review highlights animal model discoveries in the realms of prenatal immune challenges, environmental toxicants, diet, and early-life stress that have significantly advanced our understanding of the relationship between environments, epigenetic modifications, behavior and health. The translational relevance of these discoveries is addressed, as well as how animal work is informative of what researchers should study in humans. An additional theme is how human work has guided animal research, allowing scientists to delve further into the causal factors of environmental experiences and biological characteristics that contribute to abnormal brain development and disorder or disease.

**Prenatal Immune Challenges**

In the last two decades, studies provide evidence that infections during pregnancy, or maternal immune activation (MIA), lead to epigenetic and behavioral changes in offspring, with some of these same changes observed in individuals with schizophrenia and Autism Spectrum Disorder (ASD). For example, epidemiological research suggests that viral or bacterial infections during early or mid-pregnancy increase risk for neurodevelopmental disorders such as ASD and schizophrenia by 2-
to 7-fold, two disorders in which genetics is clearly involved (Brown and Patterson, 2011; Brown and Susser, 2002; Khandaker et al., 2013; Kundakovic, 2017; Meyer, Yee, and Feldon, 2007; Weber-Stadlbauer et al., 2017; Zerbo et al., 2015). Experimental models of infection have been necessary to elucidate the causal and mechanistic links between early MIA and altered neurodevelopmental and health outcomes. Such studies indeed reveal behavioral and cognitive abnormalities in adult mice and rats with prenatal exposure to, for example, bacterial endotoxin lipopolysaccharide (LPS), human influenza virus, or a viral mimic called polyriboinosinic-polyribocytidilic acid (Poly(I:C)) (Meyer, Yee, and Feldon, 2007; Weber-Stadlbauer et al., 2017; Smith et al., 2007; Wu et al., 2017). Depending on the time of exposure, rats demonstrate different cognitive and behavioral deficits, with early and mid-pregnancy exposure being the most debilitating on these outcomes (Meyer, Yee, and Feldon, 2007).

While schizophrenia and ASD are disorders with distinct characteristics, they do show similarities in social and cognitive dysfunction. For example, individuals with ASD or schizophrenia both have difficulties with social cues and social interactions. They also show deficits in prepulse inhibition (PPI) and latent inhibition (LI) (Meyer, Yee, and Feldon, 2007). Both LI and PPI are used as measures of whether an organism can filter out irrelevant stimuli. Deficits in PPI and LI indicate disrupted information processing. PPI refers to a decreased response to a startle stimulus, when the startle stimulus is preceded by a moderate neutral stimulus (Jones et al., 2008). LI refers to a decreased ability to learn the relevance of a stimulus that is
paired with an aversive or positive condition through classic conditioning if there has been a previous exposure with the stimulus in a neutral context (Lubow, 1966; Swerdlow et al., 1996). Rodents also show LI and PPI, which makes them a suitable model for testing whether and how deficits in PPI and LI reflect environmentally-driven changes in the brain. Rodents are also very social animals, enabling scientists to use them as models of social behavior. Furthermore, both schizophrenia and ASD share many of the same genes that have been identified as at-risk for the development of these disorders (Carroll and Owen, 2009; Crespi, Stead, and Elliot, 2010), and importantly, humans share almost all protein coding DNA with rodents, especially rats (Gibbs et al., 2004).

Since both human and animal model studies indicate that MIA can lead to substantial alterations in fetal brain development, often leading to behavioral dysfunction, much recent attention has focused on understanding the role of cytokines in neurodevelopment (Dahlgren et al., 2006; Hodge et al., 2007; McCullough et al., 2017; Richetto et al., 2017; Smith et al., 2007). Cytokines are substances secreted by cells of the immune system and are increased in production in response to infection (Weber-Stadlbauer et al., 2017). Rats exposed to the cytokine interleukin-6 (IL-6) in utero display deficits in PPI, LI, and social interaction (Smith et al., 2007; Wu et al., 2017), all deficits commonly observed in rodent models of ASD or schizophrenia. The mechanisms by which IL-6 does this are still largely unknown, but this cytokine crosses the placenta with more ease than others, suggesting that it could be more detrimental to neurodevelopment than other cytokines (Zaretsky et al., 2004).
Furthermore, the permeability of the placenta to IL-6 varies depending on the time of pregnancy; fetuses of rat dams who were injected in mid-pregnancy (E11-13) had higher levels of IL-6 compared to offspring of dams who were injected in late-pregnancy (E17-19) (Dahlgren et al., 2006).

Emerging evidence points towards epigenetic modifications (Hodge et al., 2007; Weber-Stadlbauer et al., 2017; Wu et al., 2017) as a route through which IL-6 influences development and behavior. Hodge et al. (2007) provided seminal in vitro evidence that IL-6 has consequences for DNA methylation. DNA methylation is the attachment of a methyl group to a cytosine on the DNA strand and is made possible by enzymes called DNA methyltransferases, or DNMTs [reviewed in 6]. DNA methylation is typically associated with gene silencing through the recruitment of corepressor proteins and the blocking of transcription factors (Doherty and Roth, 2016). In vitro, IL-6 enhances translocation of enzyme DNMT1 to the nucleus (Hodge et al., 2007), which could produce aberrant DNA methylation. But does IL-6 have this same effect in vivo and what consequences does this effect have for development? Wu and colleagues (Wu et al., 2017) investigated pathways in which IL-6 might disrupt fetal neurodevelopment by creating a knockout mouse line that lacked IL-6 receptors in the placenta (pIL-6R). Pregnant dams were exposed to Poly(I:C) during mid-pregnancy (E12.5) and expression of STAT3 in the fetal brain was measured three hours post-injection. STAT3 is a protein that gets phosphorylated by Janus kinases (JAK) in response to cytokines, acting as a transcription factor to mediate the expression of many genes. Wu et al. (2017) found that STAT3 expression was increased in the fetal
brain of wild-type mice. Furthermore, increased STAT3 activity led to increases in Myc and Stat3 expression. Increased Myc expression is observed in individuals with ASD (Ziats and Rennert, 2011), and thus animal research indicates it would be useful to explore whether individuals with ASD that have increased Myc expression also have mothers who went through an infection during pregnancy. Importantly, pIL-6R KO mice did not show an increase in IL-6 or STAT3 in the fetal brain, a finding that indicates that IL-6 receptors in the placenta are necessary for STAT3 activation and subsequent changes in gene expression in the fetal brain. MIA wild-type mice also had decreased sociality and increased anxiety-like behavior as measured by the three-chamber social and marble burying tests (Wu et al., 2017).

Viral mimetic Poly(I:C) exposure in mid- (E9) and late-pregnancy (E17) alters global methylation within the prefrontal cortex (PFC) (Richetto and Massart, 2017). Mice exposed at E9 had 2365 differentially methylated CpG sites compared to controls in adulthood. E17-exposed mice had 3361 differentially methylated sites. Methylation alterations in both E9 and E17 were enriched for genes involved in brain development, synaptic plasticity, and neuronal differentiation. Furthermore, differentially methylated genes also had corresponding altered mRNA levels, indicating that prenatal infection had a functional consequence on gene expression. Labouesse et al. (2015) also utilized a Poly(I:C) mouse model of prenatal infection to examine whether MIA affects DNA methylation, hydroxymethylation, γ-Aminobutyric acid (GABA) function and behavior. Hydroxymethylation of DNA occurs when a methylated cytosine is oxidized by ten-eleven translocation (TET)
proteins (Skortsova et al., 2016). The role of hydroxymethylation in gene regulation can vary from a transient step in the process of demethylation, to playing a stable role in epigenetic regulation (Kinde et al., 2015; Szyf and Bick, 2013; Vaissière, Sawan, and Herceg, 2008). Its role likely varies depending on developmental age, tissue type, and genomic region (Skortsova et al., 2016). MIA increases methylation and hydroxymethylation of cytosines within the PFC, specifically at \textit{GAD1} and \textit{GAD2} promoters (Labouesse et al., 2015). \textit{GAD1} and \textit{GAD2} are genes that code the GABA-synthesizing enzyme, glutamic acid decarboxylase (GAD67 and GAD65). MIA also increases binding of methyl CpG-binding protein 2 (MeCP2) at \textit{GAD1} and \textit{GAD2} promoters and decreases GAD67 and GAD65 mRNA expression.

Finally, we are learning that MIA has long-lasting behavioral and epigenetic consequences that span generations (Weber-Stadlbauer et al., 2017). Using the Poly(I:C) mouse model of MIA, Weber-Stadlbauer and colleagues (Weber-Stadlbauer et al., 2017) demonstrate decreased sociability, increased cued fear expression, and behavioral despair in third-generation mice (paternally-derived). Differentially-expressed genes involved in both glutamatergic and dopaminergic-signaling pathways may be responsible for the phenotypes. In sum, epidemiological studies informed the field of neuroscience that infection during pregnancy was likely associated with disorders such as schizophrenia and ASD. This indicated to scientists the necessity to develop rodent models of these infections to determine whether infection was causally contributing to the development of disorders. Another observation made possible because of rodent models is that of the multigenerational consequences of maternal
infection. Because of the discoveries it may be possible to develop pharmaceutical agents that block the effects of infection on the fetal brain, potentially preventing individuals from developing either schizophrenia or ASD.

Environmental Toxicants

With the increase in chemical pollutants in our society, the widespread use of plastics in everyday household items, and the use of pesticides in both mass production and local agriculture, we are increasingly exposed to chemicals that modify our epigenome (del Blanco and Barco, 2018). Bisphenol chemicals (BPA, BPAF, and BPS) and heavy metals such as lead, and pesticides (such as vinclozolin) are known to impact epigenetic and behavioral states (Nilsson and Skinner, 2015; Shi et al., 2015; Tran and Miyake, 2017). The prenatal period of development is extremely sensitive to such external factors, as it is a period of dramatic growth and differentiation of brain cells (Tran and Miyake, 2017; Kundakovic and Jaric, 2017). Particularly, neurogenesis, neural migration and maturation are processes dependent on epigenetic control, making offspring highly susceptible to the influences of bisphenol chemicals, lead, and vinclozolin (Kundakovic and Jaric, 2017). In fact, epidemiological research links these toxicants to certain epigenetic and behavioral outcomes (Tran and Miyake, 2017; Kundakovic et al., 2015).

BPA, BPAF, and BPS are classified as endocrine-disrupting chemicals (EDCs), as they bind to estrogen receptors ERα and ERβ, activating signaling cascades that lead to changes in gene expression (Bermudez, Gray, and Wilson, 2010;
Li et al., 2012; Li et al., 2018; Matsushima et al., 2010). BPAF and BPS were originally created as substitutes for BPA but have been found to have stronger effects than BPA (Li et al., 2018; Matsushima et al., 2010). In addition, both are often used in everyday products. Studies in humans indicate that BPA is detectable in urine, fetal placental tissue, cord blood, and fetal tissue (Calafat et al., 2008; Kundakovic and champagne, 2011; Mouneimne et al., 2017). To understand how bisphenol chemicals can lead to changes in the brain, Li and colleagues (Li et al., 2012) conducted an in vitro study to test the effect of BPA and BPAF on three different cell lines. Some of their observations include BPA and BPAF acting as either agonists or antagonists to ERs in a dose-dependent manner, these agents activating different genes, and these agents having effects dependent on cell line, which together are all indicative that these chemicals can disrupt endocrine signaling by way of many different mechanisms. BPS binds selectively to ERα and recruits more co-repressors of transcription than either BPA or BPAF, suggesting that BPS could have a more profound influence on gene expression, and possibly more severe effects to those who are exposed to it (Li et al., 2018). Evidence from an in vitro study shows apoptotic effects of BPAF on hippocampal neurons (Lee et al., 2013).

While in vitro studies are a great first step in understanding the consequences of toxicant exposure in a precisely controlled environment, it is important to explore their consequences under more realistic conditions where multiple cells are constantly communicating with one another and the organism is regularly interacting with its environment (i.e., in vivo). In vivo activation of ERα and ERβ leads to a host of
changes in gene expression throughout the brain. Cao et al. (2012) examined the effects of BPA on gene expression of ERα, ERβ, and Kiss1 within the hypothalamus. Neonatal rats (PN1–PN10) exposed to BPA have altered expression of all three genes. During the neonatal period, the hypothalamus undergoes steroid-directed sexual differentiation. Thus, endocrine disruption at this time could induce permanent, lifelong changes in the brain and body. The rat neonatal period is the equivalent of the third trimester in pregnancy, so future studies should examine whether high maternal exposure to BPA in late pregnancy is related to changes in the same genes of the fetus. One way to measure this is by measuring gene expression in cord blood (Kundakovic et al., 2015).

Much of the bisphenol chemical literature focuses on whether these substances activate ERs, and whether this leads to changes in gene expression. Studies are beginning to elucidate the influence of these substances on epigenetic mechanisms such as DNA methylation as driving factors of the change in gene expression and behavioral and health outcomes. For example, Dolinoy et al. (2007) investigated how maternal exposure to BPA affects DNA methylation and phenotypes of Agouti mice offspring. In utero BPA exposure yields a yellow-coated phenotype in offspring. A yellow coat phenotype occurs when the mouse is heterozygous for the agouti yellow allele. Mice with this phenotype are more prone to health problems such as obesity and diabetes (Dolinoy et al., 2007). BPA exposure also decreases DNA methylation in the Agouti gene. Hypomethylation and the yellow coat phenotype can be prevented by administering methyl donor supplements (e.g., folic acid and genistein) in the diet of
BPA-exposed dams. Kundakovic et al. (2015) also show consequences of BPA exposure on DNA methylation and do so in both rodents and humans. Male mouse offspring were most vulnerable to the effects of BPA exposure in utero, showing hypermethylation of hippocampal Bdnf (brain-derived neurotrophic factor) DNA, concomitant reduced gene expression, and behavioral deficits in a novel object recognition test (NOR). Lastly, cord blood samples from human mothers with high exposure to BPA during pregnancy likewise show hypermethylation of Bdnf DNA methylation, providing direct translational relevance of the rodent data.

Epidemiological research on BPA reveals sex-specific effects: some studies show more severe behavioral deficits such as hyperactivity and anxiety in female children exposed to BPA in utero, whereas others showed that BPA was more behaviorally disruptive to males (Tran and Miyake, 2017). It is difficult, however, to tease apart the effect of bisphenol chemicals from other factors that could contribute to the behavior of these children.

Lead exposure is severely disruptive in early development (but can also have deleterious effects when encountered throughout the lifespan). Postnatal exposure to lead is associated with increased risk for neurodevelopmental disorders and disruptions in cognition (Tran and Miyake, 2017). Lead can also cause encephalopathy at high levels (70 μg/dL, measured in blood). In fact, even at 56 μg/dL, lead leads to severe brain damage in infants (Eid and Zawia, 2016). In rodents, lead downregulates synaptic genes, lead to alterations in synapses, and increases amyloid beta 40 (aβ40) (Tran and Miyake, 2017). Increased aβ40 is especially concerning because this fibril
makes up the plaques that form in the brains of Alzheimer’s patients. Rodents exposed to lead have alterations in DNA methylation regulators (for example DNTM1 and DNTM3a), hypermethylation of numerous genes within the hippocampus, and deficits in memory formation (McCarthy et al., 2014). Interestingly, similar alterations in DNA methylation regulators following lead exposure have been observed in *Macaca fascicularis* (Richetto and Massart, 2017). Future studies could examine whether these same changes also occur in humans exposed to lead. In human embryonic stem cells, lead disrupts neuronal differentiation and induces global hypermethylation (del Blanco and Barco, 2018).

Pesticides are chemicals used to protect crops from pests, weeds, and diseases, particularly vector-borne diseases such as dengue fever or malaria (Nicolopoulou-Stamati et al., 2016). Pesticides are also used in urban green areas, sports fields, pet shampoo and building materials (Nicolopoulou-Stamati et al., 2016). Thus, the level of exposure to pesticides can vary considerably depending on a person’s environment and lifestyle. Human studies suggest that exposure to pesticides can disrupt cognition and increase the risk for cancer, asthma, diabetes, Parkinson’s disease, and leukemia (Kim, Kabir, and Jahan, 2017). Rodent studies help us realize that these health problems are attributable to pesticide exposure and can even be passed through generations (Nilsson and Skinner, 2015; Anway, Leather, and Skinner, 2006; Anway et al., 2006; Manikkam et al., 2012).

Vinclozolin is an endocrine disruptor and a common pesticide used in crops. Illustrating its damaging consequences for the epigenome and somatic health, rat pups
exposed in utero from E8-14 display symptoms of kidney disease, prostate disease, immune system abnormalities, testis and reproductive health abnormalities, and cancer (Anway, Leather, and Skinner, 2006; Anway et al., 2006). What is remarkable is that some of these health consequences are even present in fourth-generation offspring, offspring certainly never directly exposed to the pesticide. The pesticide and bug repellent mixture (permethrin and DEET) likewise has similar effects, including differential methylation patterns in the sperm of exposed males (Manikkam et al., 2012).

In sum, the dangers of gestational and early postnatal exposure to environmental toxicants is clear, and animal models have helped us realize their multigenerational consequences. These discoveries are informative for policy research aimed at reducing or preventing toxicant exposure. They are also suggestive of research avenues for strategies that could counteract the damaging effects of toxicant exposure.

**Diet**

Dietary nutrients that we ingest provide precursors and methyl groups for the process of DNA methylation. The primary source of methyl groups for DNA methylation is s-adenosylmethionine (SAM), which is catalyzed when nutrients and vitamins such as folate, vitamin B, and choline are present in the diet (Anderson, Sant, and Dolinoy, 2012; Kovacheva et al., 2007). Nutrients such as folate can alter DNA methylation in a gene and tissue-specific manner at different life stages (Pogribny et
In humans, folate, or folic acid, is the most studied regarding prenatal development (Anderson, Sant, and Dolinoy, 2012; Chidabaram, 2012), with folic acid supplementation prior to conception and during pregnancy a well-validated recommendation to significantly reduce the risk of neural tube defects (Chidabaram, 2012). Animal models have been instrumental in showing the necessity of folate for nucleic acid synthesis and methyl group bioavailability in preventing the prevalence of neural tube defects (Pogribny et al., 2008; Dolinoy, Huang, and Jirtle, 2007; Paternain et al., 2016; Wolff et al., 1998).

Intrauterine exposure to nutrient restriction likewise has epigenetic and phenotypic consequences, most famously evidenced by the Dutch Winter Famine from 1944–1945 (Brown and Susser, 2002; Heijmans et al., 2008; Hoek, Brown, and Susser, 1998; Roseboom, de Rooij, and Painter, 2006). Animal experiments provide empirical support for epigenetic mechanisms as the link between maternal diet and phenotypic outcome (Dolinoy, Huang, and Jirtle, 2007; Wolff et al., 1998). For example, scientists showed that a methyl-rich diet during pregnancy influences coat color and feeding behavior in offspring (Wolff et al., 1998). Methyl donor-supplemented diets outside of sensitive periods of development also influence DNA methylation with consequences for behavior (Paternain et al., 2016). Methyl donor supplementation for 18 weeks in adult rats can mitigate the effects of early-life stress on hippocampal DNA methylation, cholesterol levels, and forced swim behavior.
Folate and methyl-deficient diets in adulthood are also known to alter brain methylation (Pogribny et al., 2008; Kovacheva et al., 2007). Animal research also helps us see that paternal diet prior to conception has an influence on offspring development and behavior (Lambrot et al., 2013; McCoy et al., 2018). McCoy et al. (2018) depleted dietary methyl-donors of anxiety-prone male rats for five weeks prior to mating. Fathers exhibited exacerbated anxiety-like and depression-like behaviors in the open field and forced swim tests, and their offspring showed similar behavioral profiles. Lambrot et al. (2013) fed male mice a low folate diet and found that their sperm had differential methylation of genes associated with a variety of mental disorders such as schizophrenia, autism, as well as genes associated with development, cancer, and diabetes. Offspring of these males showed more birth defects.

Nutrition outside of sensitive periods of development likewise has physiological and pathological consequences, and animal work illustrates the ability of diets to produce changes in the epigenome. A chronic high-fat diet (HFD) leads to altered physiological responses to stress, an effect involving changes in gene regulatory responses regulating stress responsivity and inflammation (Sivanathan et al., 2015). An HFD can also alter methylation and gene expression within brain reward circuitry (Vucetic et al., 2012). Shen et al. (2014) induced obesity in mice via a diet in which 60% of calories came from fat. They found that diet-induced obesity (DIO) mice increased MeCP2 and DNMTs in adipose tissue at the leptin promoter region (a gene that helps regulate appetite), as well as hypoacetylation of histones and
increased binding of histone deacetylases at the same gene region. Histones are alkaline proteins that DNA wraps around and modifications to histone tails helps regulate gene activity (Zhang and Reinberg, 2001). DIO can also alter DNA methylation of memory-associated genes, including Sirt1 within the hippocampus (Heyward et al., 2016), an effect that can be reversed by resveratrol.

In sum, nutrients and diets all influence behavioral and health states of humans, with animal work making it possible for us to see how this involves changes to the epigenome. Animal work also helps us see clearly that is important to find a healthy balance, as too much or too little of nutrients can have deleterious effects. Such evidence should make individuals wary of committing long-term to particular unbalanced diets. Based on data from animals fed a high-fat diet, it would be interesting to examine the effects of the popular keto diet on DNA methylation in human participants.

**Early-Life Stress**

Stress and adversity encountered during early development are major risk factors for the development of cognitive and emotional disorders (Bolton et al., 2017; Gunnar and Quevedo, 2006; Heim and Binder, 2012; Cicchetti et al., 2016). An increasing number of studies link such developmental experiences to changes in the epigenome (Klengel et al., 2013; Nelson, 2017; Naumova et al., 2012; Romens et al., 2015). Gene systems in general that seem especially sensitive include those helping to regulate neural development, stress responsivity, and inflammation. Data gathered
from various rodent models were the driving impetus to examine DNA methylation in these cited human studies (and many others we do not have room to include) as well as non-human primate studies (Provencal et al., 2016). Animals models have been vital in providing clear experimental evidence that early caregiving environments and experiences do indeed affect DNA methylation with functional consequences for behavior, as the design of human studies and the unavoidable lack of variable control leave us unable to determine whether specific prenatal or postnatal experiences are causally responsible for the observed changes in DNA methylation, behavior, and health.

In rodents, maternal input is the major environmental experience (as fathers are typically removed from the home cage after breeding), and research with animal models has shown us that the quality of maternal care influences pup outcomes via epigenetic alterations. In a seminal study in the field of behavioral epigenetics, Weaver et al. (2004) showed that levels of maternal care (low licking/grooming (LG) vs. arched-back nursing (ABN) vs. high LG and ABN) alter methylation at the glucocorticoid receptor (GR) promoter in the hippocampus of offspring. The maternal influence on methylation (and histone acetylation) can persist into adulthood and is associated with altered stress responsivity, but importantly, is modifiable (achieved in their study via cross-foster care or HDAC inhibition). For example, adult rats who experienced low LG/ABN but were given an HDAC inhibitor had their GR expression and stress response normalized. Demonstrating translational relevance of these findings, McGowan et al. (2009) found increased methylation of NR3C1 gene (human
equivalent of GR), decreased \textit{NR3C1} expression, and decreased NGFI-A binding in the hippocampus of individuals with a history of child abuse. In similar fashion, mean \textit{NR3C1} DNA methylation levels are increased in depressed individuals with a history of abuse, with methylation at specific CG sites within \textit{NR3C1} exon 1F related to childhood emotional abuse severity (Farrell et al., 2018).

Animal models often utilize either maternal separation or limit nesting resources to simulate early adversity (Keller, Doherty, and Roth, 2018; Bockmühl et al., 2015; Ivy et al., 2008; Molet et al., 2014; Moloney et al., 2015; Roth et al., 2009). While some maternal separation studies support evidence that early-life stress affects the epigenome (Paternain et al., 2016; Moloney et al., 2015; Murgatroyd et al., 2009), others report conflicting results (Parker et al., 2006; Lehmann and Feldon, 2000; Millstein and Holmes, 2007). On the other hand, using the limited nesting resources model in rodents has proven to produce more consistent and reproducible findings across labs (Walker et al., 2017). This model has another strength: it is more naturalistic compared to maternal separation (Bolton et al., 2017). It is also relevant to humans because it simulates conditions of poverty, a very prevalent global issue (Bolton et al., 2017). Rat dams given limited nesting material have fragmented and unpredictable behaviors such as poorly-treating pups, leading to elevated corticosterone levels in the pups (Doherty et al., 2017; Gilles, Schultz, and Baram, 1996; Rice et al., 2008; Weaver et al., 2004).

Roth et al. (2009) used a limited nesting resource model to examine the effects of maternal maltreatment on the offspring’s epigenome. Their data show us that even
brief exposure to mild adversity during a sensitive period of development can increase cortical DNA methylation and suppressing gene expression (of \textit{Bdnf}). Furthermore, the experience of maltreatment alters maternal behavior (leading females to maltreat their own offspring), with some changes in DNA methylation likewise present in the next generation. Lastly, the maltreatment-induced gene changes can be mitigated with strategies that alter DNA methylation (in this study, they used a drug known to alter DNA methylation called zebularine). Keller, Doherty, and Roth (2018) recently extended these findings to show zebularine administration in adulthood can also improve some of the behavioral deficits caused by maltreatment (forced swim behavior in this study). Together, work from various animal models show that environmental or pharmacological interventions are beneficial to individuals who do not receive adequate care early in life, just like you see in the clinical literature, but provide insight into the brain mechanisms of necessary targets.

Prenatal exposure to psychosocial stress also has consequences for offspring development and health (Blaze et al., 2017; Class et al., 2014; Cao-Lei et al., 2014; Peña, Monk, and Champagne, 2012) and examples from the human literature suggest that maternal prenatal stress is related to changes in offspring methylation. For example, Cao-Lei et al. (2014) did a population-based study on the effects of the Quebec ice storm on experiences of hardship and distress of pregnant women, and how their experiences were associated with child outcomes. Thirteen years later, they observed that prenatal maternal hardship correlates with methylation levels in 1675 CpGs affiliated with 957 genes that are involved in immune function. Animal work is
what provides the causal link between prenatal stress, epigenetic modifications, and behavior. In 2008, a seminal investigation highlighted the role of epigenetic mechanisms in the maladaptive effects of prenatal stress on offspring HPA responsivity and behavior (Mueller and Bale, 2008). Blaze et al., (2017) found hypermethylation of Bdnf DNA in the medial prefrontal cortex (mPFC) of adult offspring exposed to prenatal stress (pregnant rat dams were exposed to unpredictable and variable stressors during pregnancy). Peña et al. (2012) examined prenatal stress and its effects on placental 11β-hydroxysteroid dehydrogenase type 2 (HSD11B2). HSD11B2 is an enzyme that protects the fetus from maternal stress by converting cortisol and corticosterone into inactive metabolites. However, chronic stress during pregnancy can downregulate this enzyme. Peña et al. (2012) found that prenatal stress increases methylation of HSD11B2 promoter DNA, with changes in methylation also present in the fetal hypothalamus. Together, these results provide experimental evidence of a mechanism whereby stressors in the womb can affect behavioral and health trajectories.

Lastly, animal work has shown the multigenerational transmission of epigenetic marks and behavior/health associated with developmental stress (Roth et al., 2009; Chan, Nugent, and Bale, 2018; Franklin et al., 2012; Scorza et al., 2018; Ward et al., 2013). In some instances, investigators have discovered epigenetic changes in the germline as a result of developmental exposure to stress and adversity (Franklin et al., 2010; Gapp et al., 2016; Skinner et al., 2010). These discoveries are remarkable, demonstrating a mechanism by which the effects of such experiences are
transferable across generations. They argue for better policies and practices concerning child development and health. They also help us see that early or traumatic experiences and resultant epigenetic changes should not be viewed as determinative, as there is malleability in our systems (epigenetic and brain) that can be capitalized on to improve behavior and health.

**Concluding Remarks and Recommendations for Future Research**

Without the use of animal models in science, biopsychology and behavioral neuroscience research would be largely limited to correlational studies. While correlational studies are a good starting point, they cannot establish causality. Thus, animal models are necessary to determine whether and how certain factors contribute to our biology, behavior, and health. Recent work in the field of behavioral epigenetics with various animal models has certainly started to give us a better understanding of the complex mechanisms that account for the relationship between brain development and behavioral and health outcomes. We have described evidence for how four different factors affect biology and behavior in both humans and non-human animals, and how epigenetic mechanisms mediate these outcomes (Figure 2).
Factors in the environment in addition to experiences throughout the lifespan can influence the development of an organism via epigenetic mechanisms, thus altering subsequent behavior, health, and disease outcomes. However, it is rarely the case that these factors happen exclusively. For example, children who grow up in low socioeconomic status have altered immune function (Elwenspoek et al., 2017a), are often exposed to more environmental toxicants (Tyrrell et al., 2013), suffer poor nutrition (Darmon and Drewnowski, 2008), and are exposed to various psychosocial stressors (Baum, Garofalo, and Yali, 1999). Similarly, children who experience institutionalization are affected by a pervasive mixture of these elements (Elwenspoek et al., 2017a; Troller-Renfree et al., 2018; Non
et al., 2016; Elwenspoek et al., 2017b. While the animal models reviewed in previous sections have helped us understand how prenatal immune challenges, toxicant exposure, dietary factors, and early-life stress each affect brain and behavior, future studies are certainly warranted that incorporate multiple factors to help elucidate how factors act in conjunction to influence development. Such integrative models are likely to reveal novel mechanisms with important translational relevance. As we continue to elucidate such mechanisms, we will also discover novel strategies (pharmacological and/or behavioral) to treat or even prevent certain diseases and disorders.
Chapter 2
PREVENTION OF ABERRANT DNA METHYLATION INDUCED BY CAREGIVER MALTREATMENT IN EARLY LIFE

Abstract
Poor parental care in early life often leads to maladaptive behaviors and mental health disorders. Evidence continues to mount that epigenetic mechanisms such as DNA methylation mediate the effects of poor parental care on biological and behavioral development. Pharmacological interventions are useful to test the notion that if one could potentially prevent such aberrant epigenetic activity, and if the epigenetic activity is causally related to behavioral outcome, then it should be possible to block maladaptive behavioral development from occurring altogether. This chapter describes experimental findings of administering 5-azacytidine (5-aza) concurrent with 30 minutes of caregiver maltreatment daily from postnatal (PN) days 1-7 in Long Evans rats, as a first step to determine if it is possible to prevent maltreatment-induced changes in DNA methylation. The scarcity-adversity paradigm was utilized, which employs a within-litter design where a litter of rat pups is split into three conditions. One third of the litter is exposed to a dam that displays predominately maltreatment towards pups outside the home cage (maltreatment condition). The dam displays poor maternal care because she is given little time to habituate to the experimental chamber and has limited nesting resources. Another third of the same litter is exposed to a dam that displays predominately nurturing outside the home cage (cross-foster care condition). This dam displays normal and nurturing
maternal care because she has ample nesting resources and time to habituate to the experimental chamber. The last third of the litter stays in the home cage with the biological dam in the vivarium. We found an effective dose that successfully lowered or prevented maltreatment-induced methylation. However, contrary to its successful use in the literature, the drug had some off-target effects as well as negative long-term consequences for development. Future projects will be necessary to investigate the effectiveness and safety of other pharmacological substances to combat the effects of maltreatment on DNA methylation and behavioral outcomes.

**Introduction**

The postnatal period is a time of rapid development in which animals are profoundly sensitive to environmental influences (Champoux et al., 2002; Langenhof and Komdeur, 2018; O’Mahony et al., 2009; Song and Gleeson, 2018). One such environmental influence is maltreatment, which produces significant changes in brain and behavioral development (Bolton et al., 2017; Cicchetti et al., 2016; Gunnar and Quevedo, 2006; Nelson, 2017). Indeed, childhood maltreatment is a significant risk factor for the later development of mental health disorders such as depression, anxiety, post-traumatic stress disorder, and bipolar disorder (Cicchetti and Toth, 2005; Gil et al., 2009; Heim and Binder, 2012; Stein, Schork, and Gelernter, 2008; Teicher and Samson, 2013).

One way that childhood maltreatment can affect development is through epigenetic changes to the genome. Epigenetics is a term used to describe biological
mechanisms that evoke changes in gene activity without modifying the underlying DNA sequence (Doherty and Roth, 2016; Peschansky and Wahlestedt, 2014). These changes, triggered by various environmental factors, can alter subsequent biological activity and behavior (Doherty and Roth, 2016; Szyf and Bick, 2013). While there are different types of epigenetic mechanisms, this study focused on DNA methylation. DNA methylation is when a methyl group is attached to cytosines of the DNA strand via DNA methyltransferases (DNMTs). DNA methylation is often associated with repressed gene transcription (Blaze and Roth, 2013; Siegfried et al., 1999). As evidenced by previous work in rodents, brain DNA methylation mediates adult behavior following exposure to differential maternal care (see Lutz and Turecki, 2014 for review; Roth et al., 2009; Weaver et al., 2004). Further, DNA methylation is also altered after experiences of maltreatment in early life in monkeys (measured in blood mononuclear cells; Capitanio et al., 2005) and in humans (in the hippocampus; McGowan et al., 2009). Differential maternal care also influences corticosterone concentrations measured in plasma as well as methylation and expression of glucocorticoid receptor promoter, indicating alterations of the hypothalamic-pituitary-adrenal (HPA) axis (Weaver et al., 2004).

Our lab uses a rodent model to study the relationship between maltreatment, epigenetic changes, and behavioral development. We previously showed that maltreatment induced changes in methylation within the (Brain-derived neurotrophic factor) Bdnf gene in the prefrontal cortex (PFC) of rats (Roth et al., 2009). BDNF is a protein that is crucial for brain development and plasticity (Cohen-Cory et al., 2010;
Bathina and Das, 2015). Its malfunction is implicated in various psychological disorders prominent in individuals with a history of childhood maltreatment (see Autry and Montaggia, 2012 for review; Kundakovic et al., 2015). Briefly, maltreatment was associated with higher methylation in exon IX of Bdnf, a relationship seen across development. Decreased gene expression was also apparent in adulthood. A causal link was established between early life experiences and changes in the epigenome when administration of the DNMT inhibitor (DNMTi) zebularine normalized methylation and Bdnf mRNA levels in maltreated rats (Roth et al., 2009). These findings also importantly hinted that alterations in the brain and behavior induced by maltreatment are not necessarily permanent but are potentially reversible or preventable.

Studies have found that different epigenome-modifying drugs such as trichostatin A (Hawk, Florian and Abel, 2011; Weaver et al., 2004; Weaver, Meaney and Szyf, 2006) valproic acid (VPA; Tremolizzo et al., 2005), and sodium butyrate (Schroeder et al., 2007) can improve memory (Hawk et al., 2005; Weaver et al., 2004) and anxiety- and depressive-like behavior (Tremolizzo et al., 2005; Weaver et al., 2006) when administered to adult rodents. However, few studies have examined whether aberrant changes in the epigenome can be pharmacologically prevented in early life, rather than rescued in adulthood. Because early life experiences set the course for subsequent biological and behavioral development, it may be possible to prevent maladaptive outcomes by administering pharmacological intervention at the same time as the occurrence of early stressors. More recently, Kao et al., 2012 tested
whether VPA or 5-aza-2’-deoxycytidine (5-aza) given at the time of early life maternal separation, could prevent methylation and behavioral changes induced by early life maternal separation (MS) and found that VPA reversed changes in fear response in adolescence, as well as changes in histone methylation. Durenkova, Aleksandrova, and Zarayskaya (2019) also showed the preventative effects of VPA on the damaging effects of MS on maternal behavior.

The present study aimed to expand on the previous findings of Roth et al. (2009) by exploring whether changes in methylation can be pharmacologically prevented in early life rather than pharmacologically reversing them in adulthood. This is an important first step in a line of research in the lab working to establish causality between maltreated-induced methylation changes and maladaptive behavioral outcomes. Because zebularine does not cross the blood-brain barrier, we used the drug 5-aza (also a DNMTi), a cytidine analogue crosses the blood-brain barrier and incorporates into DNA (Chabot, Rivard, and Momparler, 1983).

Methods

Subjects
All animal procedures were conducted with approval of the University of Delaware Animal Care and Use Committee and in accordance with NIH guidelines. Long-Evans rats from Envigo were bred in-house and kept in a temperature-controlled room on a 12-hr light/dark cycle. Animals were housed in polypropylene cages with wood shavings and had *ad libitum* access to food and water. Dams (multiparous, from
90 days to 1 year old) were used to generate experimental litters and to act as stimulus dams. Breeder males and females were only paired together once to ensure genetic diversity of the experimental litters. On postnatal day (PN) 1, pups were culled to either 9 or 12, with an equal number of males and females whenever possible within each litter.

**Infant Manipulations**

We utilized the scarcity-adversity model in our experiment (as previously described in e.g. Roth et al., 2009; Doherty et al., 2017; Keller, Doherty and Roth, 2018). This model employs a within-litter design in which a litter of pups is split into three conditions for 30 minutes daily from PN1-PN7. One third of the litter is exposed to a stressed dam outside the home cage (maltreatment, or MAL condition). The dam displays predominantly adverse maternal care because she is given insufficient time to habituate to the testing chamber and has limited nesting resources (100 ml of bedding). Another third of the litter is exposed to a non-stressed dam outside the home cage (cross-foster care, or CFC condition). This dam displays predominately nurturing and normative care because she has ample nesting resources (2cm layer on chamber floor) and 1 hour to habituate to the testing chamber. The last third of the litter stays in the home cage with the biological dam within the vivarium (normal maternal care, or NMC condition). Pups of the stimulus dams were placed in an incubator during infant manipulations. All pups were immediately returned to their home cages following the 30 minutes of manipulation. All stimulus dams were lactating and
matched in post-partum age and diet to the biological mother of experimental pups, as previous research showed that pups cannot distinguish between dams of the same post-partum age and fed the same diet (Leon, 1975). Manipulations were conducted during the light cycle (7AM to 7PM). A total of 35 litters were generated and used in this study. There were 2-4 siblings per group, so a litter average was calculated for siblings in the same group.

To confirm the efficacy of this model, videos of maternal behavior in all three conditions were recorded and scored independently by two separate observers. Nurturing behaviors (licking, nursing, grooming pups), along with adverse behaviors (stepping, dragging, roughly handling), were recorded once in every 5-minute time bins. Vocalization recordings were also collected during testing as a measure of distress and were scored by two separate observers.

After the 7th day of manipulations, pups were left undisturbed with their biological mothers until PN8 (for early life biochemical analysis) or weaning at PN21-23 (for early adolescence biochemical analysis conducted at PN30). At weaning, pups were placed in cages of two or three same-sex littermates until PN30.

Drug Administration
Pups were injected intraperitoneally daily immediately prior to PN1-PN7 manipulations. Control animals received either 0.5 mg/kg VEDCO Inc. saline solution or 1.0 mg/kg VEDCO Inc. saline solution, while animals in the drug treatment received 0.5 mg/kg 5-aza (from Sigma-Aldrich) dissolved in VEDCO Inc. normal
saline solution, or 1.0 mg/kg 5-aza dissolved in VEDCO Inc. normal saline solution. We chose to use 5-aza as this drug was previously tested in rat pups (at a much higher dose of 5 mg/kg) from PN2-PN9 at alternating days, and 10 mg/kg at PN5 and PN9 with no evidence of toxicity (Kao et al., 2012).

Sample Collection (Brain and Plasma)
Animals were sacrificed via rapid decapitation at PN8. At PN30, animals were sedated with isoflurane (VEDCO Inc. IsoSol™) prior to decapitation. Brains were extracted, the PFC was isolated, and was homogenized in 350 µL lysis buffer. We focused on the PFC for symmetry with previous work in the lab (Roth et al., 2009). Peripheral blood was collected in a microcentrifuge tube with 50 µL EDTA and centrifuged at 12,700 rpm in 4°C for 15 minutes to separate plasma from red blood cells. A pipet was used to extract the separated plasma and place it in a new tube. Plasma was collected in order to obtain a peripheral measure of stress. Plasma was used to analyze levels of corticosterone, a hormone involved in stress responses in rodents.

DNA/RNA Extractions and Biochemical Analyses
DNA and RNA were extracted from the homogenized PFC by using Qiagen (Valencia, CA) AllPrep DNA/RNA Mini Kit according to manufacturer’s protocol. Concentrations of DNA (eluted to 50 ug/ul) and RNA (eluted to 30 ug/ul) were obtained via spectrophotometry. For gene-specific methylation, bisulfite conversion
and cleanup of DNA was conducted by using Qiagen (Valencia, CA) EpiTect Bisulfite Kit according to manufacturer’s protocol. Next, bisulfite DNA was used for methylation-specific real-time PCR (MSP) of methylated BDNF IX (Bio-Rad CFX96 system). Primers designed to distinguish methylated sites were used to amplify bisulfite-modified DNA associated with BDNF IX exon (Doherty, Forster and Roth, 2016; Blaze, Schueing, and Roth, 2013; Roth et al., 2009). Tubulin was used as a reference gene and all samples were run in triplicates. The $2^{-\Delta\Delta C_q}$ method was used to calculate the relative methylation for the gene of interest relative to the reference gene as previously described in Livak and Schmittgen (2001). DNA was also used to measure global methylation via Epigentek (Brooklyn, NY) MethylFlash™ Global DNA Methylation (5-mC) ELISA Easy Kit according to manufacturer’s protocol. Absorbance was measured using the Infinite® F50 microplate reader (Tecan, Männedorf, Switzerland) with the amount of 5-mC DNA being proportional to the intensity of the optical density (method previously described in Doherty, Forster, and Roth, 2016).

Total RNA was used for cDNA synthesis via Qiagen QuantiTect Reverse Transcription Kit according to manufacturer’s protocol. cDNA was amplified via RT-PCR (Blaze et al., 2017) using Taqman probes (ThermoFisher Scientific, Grand Island, NY) to target transcripts containing DNMT1, DNMT3a and DNMT3b. Tubulin was used as a reference gene. The $2^{-\Delta\Delta C_q}$ method was used to calculate the relative gene expression for each gene of interest relative to the reference gene as previously described in Livak and Schmittgen (2001).
Plasma Analysis
Corticosterone concentration in plasma was measured by using Arbor Assays® (Ann Arbor, MI) corticosterone enzyme immunoassay kit according to manufacturer’s protocol. This kit uses antibodies to detect corticosterone in plasma, which is measured via absorbance at 450nm with Infinite® F50 microplate reader (Tecan, Männedorf, Switzerland). My Assays®, an online program, was used to process raw data obtained from the corticosterone assay.

Statistical Analyses
Overall maternal behavior was analyzed using a two-way ANOVA (factors: type of behavior and condition) and Tukey's post hoc tests when appropriate. There were seven types of behaviors: step on, drop, drag, actively avoid, rough handle, lick/groom, and nurse/hover. Individual maternal behaviors were analyzed with a one-way ANOVA and Tukey's post hoc tests when appropriate. Vocalization data were analyzed via a one-way ANOVA. MSP data were analyzed by a two-way ANOVA (factors: condition and drug) and Fisher’s LSD post-hoc test when appropriate. We used Fisher’s test for these data for symmetry with a previous study and because we were only interested in a few specific planned comparisons. Global methylation data were analyzed via a two-way ANOVA (factors: condition and drug). Gene expression data were analyzed using a two-way ANOVA (factors: condition and drug) and Tukey’s post hoc tests when appropriate. A three-way ANOVA (factors: condition, drug, sex) was conducted for corticosterone analysis, followed by Tukey’s post hoc
tests when appropriate. A Mantel-Cox log-rank survival test was conducted to compare survival between saline and 5-aza pups.

**Results**

Maternal Behavior and Vocalizations

PN8 Cohort

Consistent with previous findings (Blaze, Scheuing, and Roth, 2013; Doherty, Forster, and Roth, 2016; Roth et al., 2009), there were more instances of aversive caregiving behaviors in the MAL condition relative to the NMC and CFC conditions and more instances of nurturing behaviors in the NMC and CFC conditions relative to the MAL condition (Tables 1 and 2). There was an interaction of type of behavior (aversive vs. nurturing) and condition (NMC, CFC, MAL), $F(2, 60) = 53.84, p < .0001$. There was a main effect of type of behavior, $F(2, 60) = 280.9, p < .0001$.

Table 1  PN8 cohort: overall nurturing vs. aversive behaviors experienced from PN1-PN7.

<table>
<thead>
<tr>
<th></th>
<th>2-way ANOVA</th>
<th>NMC</th>
<th>CFC</th>
<th>MAL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$F$</td>
<td>$DFn$, $DFd$</td>
<td>$p$</td>
<td>$M$</td>
</tr>
<tr>
<td>Interaction</td>
<td>53.84</td>
<td>2, 60</td>
<td>&lt;.0001</td>
<td>-</td>
</tr>
<tr>
<td>Type of Behavior</td>
<td>280.9</td>
<td>2, 60</td>
<td>&lt;.0001</td>
<td>-</td>
</tr>
<tr>
<td>Aversive</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.218</td>
</tr>
</tbody>
</table>
The one-way ANOVA was significant for “step-on”, \( F(2, 30) = 17.63, p < .0001 \). “Step-on” was an adverse behavior that occurred significantly more in the MAL condition compared to NMC \( (p = .0001) \) and CFC \( (p < .0001) \). The one-way ANOVA was also significant for “actively avoid”, \( F(2, 30) = 10.39, p = .0004 \), for “rough handle”, \( F(2, 30) = 18.55, p < .0001 \), for “lick/groom”, \( F(2, 30) = 4.985, p = .0135 \), and for “nurse/hover”, \( F(2, 30) = 16.48, p < .0001 \). MAL dams actively avoided their pups more than NMC \( (p = .0009) \) and CFC \( (p = .0017) \) dams and roughly handled pups significantly more than CFC dams \( (p < .0001) \). Both NMC and CFC dams exhibited more nurturing behaviors such as licking and grooming than MAL dams \( (\text{NMC}: p = .0314, \text{CFC}: p = .0238) \) and nursing/hovering over pups \( (\text{NMC}: p = .0004, \text{CFC}: p < .0001) \). Audible and ultrasonic vocalizations were also recorded and scored as a measure of distress. There were no significant differences in vocalizations across groups (Table 3).

### Table 2

PN8 cohort: results of one-way ANOVA, means and standard errors of each individual aversive or nurturing behavior.

<table>
<thead>
<tr>
<th>Nurturing</th>
<th>-</th>
<th>-</th>
<th>-</th>
<th>0.905</th>
<th>0.032</th>
<th>0.951</th>
<th>0.015</th>
<th>0.642</th>
<th>0.071</th>
</tr>
</thead>
<tbody>
<tr>
<td>One-way ANOVA</td>
<td>NMC</td>
<td>CFC</td>
<td>MAL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( F )</td>
<td>( DF_n, DF_d )</td>
<td>( p )</td>
<td>( M )</td>
<td>( SE )</td>
<td>( M )</td>
<td>( SE )</td>
<td>( M )</td>
<td>( SE )</td>
<td></td>
</tr>
</tbody>
</table>
Table 3  PN8 cohort: results of one-way ANOVA of audible and ultrasonic vocalizations.

<table>
<thead>
<tr>
<th>Behavior</th>
<th>NMC</th>
<th>CFC</th>
<th>MAL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>p</td>
<td>M</td>
</tr>
<tr>
<td>Audible</td>
<td>0.476</td>
<td>.6277</td>
<td>38.45</td>
</tr>
<tr>
<td>Ultrasonic</td>
<td>0.538</td>
<td>.5916</td>
<td>54.93</td>
</tr>
</tbody>
</table>

PN30 Cohort
Consistent with previous findings (Blaze, Scheuing, and Roth, 2013; Doherty, Forster, and Roth, 2016; Roth et al., 2009) and PN8 data, there were more instances of aversive caregiving behaviors in the MAL condition relative to NMC and CFC conditions and more instances of nurturing behaviors in the NMC and CFC conditions.
relative to the MAL condition (Tables 4 and 5). There was an interaction of type of behavior (nurturing vs. aversive) and condition (NMC, CFC, MAL), $F(2, 18) = 76.01$, $p < .0001$. There was a main effect of type of behavior, $F(1, 18) = 389.8, p < .0001$.

Table 4  
PN30 cohort: overall nurturing vs. aversive behaviors experienced from PN1-PN7.

<table>
<thead>
<tr>
<th></th>
<th>2-way ANOVA</th>
<th></th>
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<tr>
<td></td>
<td>$F$</td>
<td>$DFn, DFd$</td>
<td>$p$</td>
<td>$M$</td>
<td>$SE$</td>
<td>$M$</td>
<td>$SE$</td>
</tr>
<tr>
<td>Interaction</td>
<td>76.01</td>
<td>2, 18</td>
<td>$&lt;.0001$</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Type of Behavior</td>
<td>389.8</td>
<td>1, 18</td>
<td>$&lt;.0001$</td>
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<td>-</td>
<td>-</td>
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<tr>
<td>Aversive</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.213</td>
<td>0.026</td>
<td>0.128</td>
<td>0.038</td>
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<tr>
<td>Nurturing</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.895</td>
<td>0.031</td>
<td>0.978</td>
<td>0.013</td>
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The one-way ANOVA was significant for “step-on”, $F(2, 9) = 44.39, p < .0001$. “Step-on” occurred significantly more in the MAL condition compared to NMC ($p < .0001$) and CFC ($p < .0001$). The one-way ANOVA for “actively avoid” was significant, $F(2, 9) = 9.618, p = .0058$, and Tukey’s post hoc tests showed there were significantly more instances of “actively avoid” in the MAL than in NMC or CFC ($p = .0106$ for both). The one-way ANOVA was significant for “lick/groom” was also significant, $F(2, 9) = 13.77, p = .0018$. NMC dams exhibited more licking and grooming than MAL and CFC dams (MAL: $p = .0231$, CFC: $p = .0015$). There were no significant differences in vocalizations across groups for PN30 (Table 6).
Table 5  PN30 cohort: results of one-way ANOVA, means and standard deviations of each individual aversive or nurturing behavior.

<table>
<thead>
<tr>
<th>One-way ANOVA</th>
<th>NMC</th>
<th>CFC</th>
<th>MAL</th>
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</thead>
<tbody>
<tr>
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<td>$p$</td>
<td>$M$</td>
</tr>
<tr>
<td><strong>Step on</strong></td>
<td>44.39</td>
<td>2, 9</td>
<td><strong>&lt;.0001</strong></td>
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<td><strong>Drop</strong></td>
<td>3.522</td>
<td>2, 9</td>
<td>.0742</td>
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<tr>
<td><strong>Drag</strong></td>
<td>1.296</td>
<td>2, 9</td>
<td>.3202</td>
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<tr>
<td><strong>Actively avoid</strong></td>
<td>9.618</td>
<td>2, 9</td>
<td><strong>.0058</strong></td>
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<td><strong>Rough handle</strong></td>
<td>3.858</td>
<td>2, 9</td>
<td>.0617</td>
</tr>
<tr>
<td><strong>Lick/groom</strong></td>
<td>13.77</td>
<td>2, 9</td>
<td><strong>.0018</strong></td>
</tr>
<tr>
<td><strong>Nurse/hover</strong></td>
<td>3.904</td>
<td>2, 9</td>
<td>.0602</td>
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Table 6  One-way ANOVA results for PN30 audible and ultrasonic vocalizations.

<table>
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<th>CFC</th>
<th>MAL</th>
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</thead>
<tbody>
<tr>
<td>$F$</td>
<td>$p$</td>
<td>$M$</td>
<td>$SE$</td>
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<tr>
<td><strong>Audible</strong></td>
<td>1.819</td>
<td>.2042</td>
<td>45.81</td>
</tr>
<tr>
<td><strong>Ultrasonic</strong></td>
<td>3.105</td>
<td>.0819</td>
<td>67.93</td>
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40
Because there was no significant difference in methylation between normal care (NMC) and cross-foster care (CFC) pups, we combined them into a “nurturing” (NUR) category. We also collapsed across sex because there were no significant differences between males and females in any groups. NMC and CFC pups did not differ in methylation regardless of the drug treatment (saline, 0.5 mg 5-aza or 1.0 mg 5-aza; see figure 3). A two-way ANOVA revealed that there was no interaction of drug and infant condition, $F(2, 122) = 2.313, p = .1033$. There was no main effect of drug, $F(2, 122) = 1.563, p = .2136$. However, there was a main effect of infant condition, $F(1, 122) = 7.291, p < .01$, with MAL pups showing higher methylation than NUR controls. Fisher’s LSD post-hoc planned comparisons showed that maltreated pups who received saline had more methylation than NUR/SAL controls. However, MAL/1.0 mg 5-aza pups were not different from NUR/SAL controls and had significantly less methylation than MAL/SAL pups ($p < .05$, Figure 3A). Because of this finding, we decided to proceed with the 1.0 mg dose of 5-aza for the next phase of the experiment.
A) PN8 MSP results for *bdnf* IX indicate that there is a main effect of condition. Error bars represent SEM. *n = 19-23/group. NUR = nurturing and MAL = maltreatment. **p<0.01 MAL vs. NUR; #p<0.05 MAL/SAL vs. NUR/SAL and MAL 1.0 mg.*

B) PN8 global methylation results were not significant. *n = 12-14/group.*
PN8 Global Methylation

Because 5-aza is nonspecific, we also measured global methylation to determine if the drug was significantly altering methylation across the epigenome within the PFC. Suggestive of some specificity of our results presented in Figure 3, there were no significant differences in the global methylation (see Figure 3B).

PN8 Gene Expression

Because the drug is known to interfere with DNMT activity and could lead to aberrant DNMT regulation of various genes important for development at a period of rapid neuronal development (Klintsova, Hamilton, and Boschen, 2013), we measured gene expression of DNMTs (DNMT1, DNMT3a, and DNMT3b) in the PFC. Because there were no significant differences between sex in DNMT1 PN8 data, they were collapsed within each drug treatment and within conditions (NMC, CFC, MAL). A two-way ANOVA revealed that there was no interaction between infant condition and drug, $F(2, 68) = 2.784, p = .0689$. There was no main effect of drug, $F(1, 68) = 0.4093, p = .5245$ on gene expression of DNMTs. There was a main effect of condition, $F(2, 68) = 20.35, p < .0001$ (see Figure 4A). Tukey’s comparisons showed that CFC pups had higher DNMT1 expression than NMC pups ($p = .0016$), and MAL pups had higher DNMT1 expression than NMC pups ($p < .0001$) and CFC pups ($p = .0142$). DNMT3a PN8 data were collapsed across sex because they did not significantly differ for males and females. There was also effect of condition for DNMT3a, $F(2, 69) = 12.99, p < .0001$ (see Figure 4B). Tukey’s multiple comparisons test showed that CFC pups had higher DNMT3a expression than NMC pups ($p =
and MAL pups also had higher DNMT3a expression than NMC pups ($p < .0019$). DNMT3b PN8 data was collapsed across sex because they did not significantly differ for males and females. There was a main effect of condition, $F(2, 66) = 9.872$, $p = .0002$ (see figure 4C), with Tukey’s multiple comparisons test showed that MAL pups had higher DNMT3b expression than NMC pups ($p < .0001$) and CFC pups ($p = .0108$).
Figure 4  PN8 gene expression of DNMTs indicate a main effect of condition across all three DNMTs. Error bars represent SEM. NMC = normal maternal care, CFC = cross-foster care, MAL = maltreatment. A) \( n = 11-13 \) /group. * designates NMC vs. MAL and CFC vs. MAL. # designates NMC vs. CFC. B) \( n = 11-13 \) /group. * designates NMC vs. CFC: * and NMC vs. MAL. C) \( n = 10-13 \) /group. * designates NMC vs. MAL and CFC vs. MAL.
PN8 Corticosterone Assay

Corticosterone (CORT) was also analyzed as a peripheral measure of stress.

Because there was no significant difference between NMC and CFC groups, we collapsed the two into the NUR category. For PN8 data, a three-way ANOVA was conducted (Sex × Drug × Condition). There was a significant three-way interaction, $F(1, 76) = 9.872, p = .0234$. There was no main effect of condition, $F(1, 76) = 0.024, p = .8771$, or sex, $F(1, 76) = 3.057, p = .0844$. However, there was a main effect of drug, $F(1, 76) = 23.26, p < .0001$. There was an interaction of condition × sex, $F(1, 76) = 6.554, p = .0125$, but not of condition × drug, $F(1, 76) = 0.128, p = .7210$, or drug × sex, $F(1, 76) = 0.770, p = .3831$. Tukey’s multiple comparisons test showed that MAL males and females who received 5-aza were significantly different from one another ($p = .009$; see Figure 5). MAL/5-aza male pups were also significantly different from MAL/SAL females ($p = .004$), MAL/SAL males ($p = .0046$), NUR/SAL females ($p < .0001$), and NUR/SAL males ($p = .0004$). Lastly, NUR/5-aza females were significantly different from NUR/SAL females ($p = .0110$) and NUR/SAL males ($p = .036$).
Figure 5  PN8 CORT concentration multiple comparisons results. NUR = nurturing and MAL = maltreatment. Error bars represent SEM. \( n = 19-25 \)/group. * designates MAL/5-aza/males vs. MAL/5-aza/females, MAL/5-aza/males vs. MAL/SAL pups, and MAL/5-aza/males vs. NUR/SAL pups. # designates NUR/5-aza/females vs. NUR/SAL pups.

PN30 Survival Analysis
While there were no signs of toxicity at PN8, 1.0 mg 5-aza pups tended to be visibly smaller and frailer than their saline counterparts from PN10-30. We also observed increased mortality for 5-aza pups (27 out of 38 pups). This outcome was unexpected, as previous research used much higher doses of the drug in pups at the same age and did not report any issues with subsequent toxicity or mortality (Kao et al., 2012). Out of 33 saline pups, only two died unexpectedly. Mantel-Cox log-rank survival test was conducted to compare survival between saline and 5-aza pups. The survival curves were significantly different, \( \chi^2 (1) = 31.42, p < 0.0001 \), suggesting that
5-aza treatment was toxic to pups (see Figure 6). We conducted chi squares to determine if there was an effect of sex or condition, but neither sex nor condition were significant, $\chi^2 (1) = 2.94, p = 0.0867, \chi^2 (1) = 3.27, p = 0.1952$, respectively.

**Figure 6** Survival analysis results. Survival rate was significantly different between drug treatment and saline groups ($p < .0001$).

PN30 Methylation-Specific RT-PCR
We measured methylation of *Bdnf* IX DNA in adolescence (PN30) to determine if the methylation levels found in PN8 would persist into later life stages or if methylation would change with development. We were unable to complete data collection for this time point because of unexpected drug toxicity but nonetheless summarize data for subjects we could gather data from in Figure 7.
PN30 Global Methylation
Because of the small sample sizes in the PN30 data, statistical analyses could not be conducted. Descriptive results can be seen in figure 7.

**Figure 7**  PN30 MSP and global methylation descriptive results. NUR = nurturing and MAL = maltreatment. NUR category is NMC and CFC collapsed to match PN8 data. Error bars represent SEM. For both panels, n=3-6/group.
PN30 Gene Expression

We also measured PN30 gene expression of DNMTs (DNMT1, DNMT3a, and DNMT3b) in the PFC. As mentioned before, since the drug was administered at a period of rapid neuronal development (Klintsova, Hamilton, and Boschen, 2013), it is possible that 5-aza altered normal functioning of DNMTs, leading to aberrant DNMT regulation of various genes important for development. This, in turn, could have led to proliferation of abnormal cells, which could contribute to fatality. Because of the small sample sizes in the PN30 data, statistical analyses could not be conducted. Descriptive results can be seen in Figure 8.
Figure 8  PN30 DNMT gene expression descriptive results. Error bars represent SEM. NMC = normal maternal care, CFC = cross-foster care, MAL = maltreatment. For all panels, n=3-6/group.
PN30 Corticosterone Assay

We also conducted the CORT assay with PN30 plasma. Because of the small sample sizes in the PN30 data, statistical analyses could not be conducted. Descriptive results can be seen in Figure 9.

Figure 9  PN30 descriptive results for CORT. Error bars represent SEM. NMC = normal maternal care, CFC = cross-foster care, MAL = maltreatment. n = 0-5/group.

Discussion

The model we used to elicit maltreatment produced consistent behavioral findings with our previous work (Blaze, Scheuing, and Roth, 2013; Doherty, Forster, and Roth, 2016; Keller, Doherty, and Roth, 2018; Roth et al., 2009), as well as those
of others who implemented scarce resources to elicit adverse or aberrant caregiving behaviors (Doherty et al., 2017; Ivy et al., 2008). MAL dams exhibited more adverse care than NMC and CFC dams. NMC and CFC dams also exhibited more nurturing behaviors than MAL dams. We also replicate an observation regarding methylation previously reported (Roth et. Al., 2009). Maltreated pups who received saline had significantly higher methylation than nurtured pups for Bdnf IX DNA in the PFC. These results support the continued use of this model for examining the effects of early life stress on epigenetic mechanisms. They are also consistent with findings of other stimuli and early adverse environmental conditions altering methylation of Bdnf (Blaze, Schueing, and Roth, 2013; Kundakovic et al., 2015; Roth et al., 2009).

We found that a 1.0 mg/kg dose of 5-aza successfully normalized methylation of Bdnf IX DNA in both maltreated males and females. The observation that a pharmacological agent can normalize DNA methylation associated with maltreatment is consistent with previous work in our lab (Roth et al., 2009), but here we now show a pharmacological approach capable of normalizing methylation and/or combatting the effects of maltreatment earlier in development. Previous research shows that 5-aza can degrade DNMT1 in vitro (Patel et al., 2010) and downregulate DNMTs 3a and 3b when injected directly into the medial PFC (mPFC; Qiao et al., 2017) thus we decided to measure DNMT gene expression in the PFC to determine if the drug was altering their expression. We found that while the drug was not affecting DNMT expression, there was an effect of infant condition. Generally, rats in the maltreatment group had
higher gene expression of all DNMTs relative to nurturing groups, which is consistent with the observation of higher methylation in that group.

We measured plasma corticosterone concentration to determine if the drug treatment and/or conditions altered the stress response, as one way to visualize off-target effects of the drug. We found a sex-specific effect of drug and condition. MAL/5-aza males had significantly higher CORT than NUR/SAL males, females, and MAL/SAL males and females. Interestingly, NUR-5-aza females had significantly higher CORT than NUR/SAL males and females. Previous work shows that sexes differ in epigenetic patterns as early as birth (see McCarthy et al., 2009 for review). Thus, 5-aza could have had differential effects on methylation patterns of hormones of both sexes. SAL animals across conditions had relatively low CORT, which is consistent with the stress hyporesponsive period of development. Specifically, from PN4-14, rat pups show a blunted CORT response to stressors in the presence of a caregiver (see Levine, 2002; Moriceau, Roth, and Sullivan, 2010 for review). CORT levels are however dramatically increased when pups are separated from their mothers, as evidenced by previous research (Stanton, Gutierrez, and Levine, 1988; Stanton and Levine, 1988; Stanton and Levine, 1990). It is possible that 5-aza was altering methylation and/or expression of genes that regulate CORT, thus interfering with the protective effect of a caregiver's presence.

Unlike our previous work where we have shown the occurrence of more ultrasonic vocalizations from pups in the maltreatment condition (Blaze et al., 2013; Doherty et al., 2017; Roth et al., 2014), here we did not see significant differences in
vocalizations across infant conditions. Vocalizations are used as a measure of distress, as rat pups from PN4-10 are known to emit 40kHz vocalizations when separated from their mothers or experience some other form of adversity (Litvin, Blanchard, and Blanchard, 2010; Portfors, 2007). In this current study, there were many instances of vocalizations across all three conditions but given the design of the experimental chamber and position of recording devices we were unable to distinguish and quantify vocalizations between saline- and drug-treated pups within each condition. It is certainly possible that 5-aza changed vocalization rates and confounded the vocalization data.

When we tried to grow up the animals to determine if normalized methylation persisted into later life, we discovered the drug was having toxic effects that were not apparent at PN8. This was completely unexpected, as previous research tested higher doses of the drug and did not report any issues with toxicity or mortality (Kao et al., 2012). Specifically, Kao et al. (2012) administered either 5.0 mg/kg 5-aza every other day from PN2-9 or 10.0 mg/kg 5-aza at PN4 and PN9 in conjunction with MS and allowed animals to reach adulthood before running their animals (vehicle- and drug-treated) in behavioral tests. There were no fatalities reported in their manuscript and the authors were able to complete their study for analysis of adult behavioral data. We ran a survival analysis to confirm that the drug was driving mortality, and indeed found that the drug was toxic to pups.

Since we administered the drug intraperitoneally, it is possible it altered the epigenome across the whole organism (i.e. in additional organs than the brain),
throwing off major developmental and cell proliferation processes in general. The CORT data collected on PN8 animals, and what we do have from the PN30 animals, do suggest one off-target effect of the drug involves this system. One could also speculate that administering the drug every day could be an issue, depending on the drug’s half-life. The beta half-life of 5-aza in adult humans is 15-25 minutes (Momparler, 2005), in kids 10-15 (Momparler, 2005), and in rabbits and dogs, the alpha half-life is 5 minutes (Chabot et al., 1983). To our knowledge, there is no information on its alpha or beta half-life in rats. However, because of its short half-life across various species, it is possible that the half-life is not much different in rats. It is unlikely that the daily dose administration was the problem; however, our daily administration differs from the every-other-day regimen used in the Kao et al. study.

In conclusion, we were able to provide preliminary evidence that it may be possible to counteract the effects of maltreatment on DNA methylation in the infant PFC. 5-aza proved to have some unexpected developmental consequences and would not provide a viable strategy to test its ability to block maladaptive behavior outcomes. It will be thus necessary and worthwhile in future research to investigate other epigenome-modifying drugs to establish safe and effective alternatives. Understanding the relationship between maltreatment, DNA methylation, and behavioral outcomes promises to advance our understanding of how early adversity influences the development of behavior and biological targets for efforts to improve the health of those affected by early adversity.
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Appendix A

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Appendix B

IACUC APPROVAL

Form is attached below.

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IACUC Approval Signature: [Signature]

Date of Approval: 2/19