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Early-life Programming of Emotional Behaviors and Cardiovascular Function

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EARLY-LIFE PROGRAMMING OF EMOTIONAL BEHAVIORS AND CARDIOVASCULAR FUNCTION

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CELL, MOLECULAR AND DEVELOPMENTAL BIOLOGY

ABSTRACT

Extensive evidence implicates bi-directional relationship between mood disorders and cardiovascular disorders. Early-life experience can have strong effects both on emotional development and cardiovascular function throughout life. Studies in humans are limited to correlational analyses, which are necessarily limited in terms of revealing mechanistic underpinnings of these associations. Thus, various pre-clinical models are utilized to investigate the effects of early-life experience in various domains, such as behavior and cardiovascular function, which are likely mediated by epigenetic mechanisms.

Previous studies have used maternal separation and neonatal handling in developing rodents as a way to model differences in early-life experience. The effect of early-life maternal separation has been found to be adaptive as well as maladaptive. Cumulative stress hypothesis favors the maladaptive consequence of maternal separation, which states that an individual is more likely to be vulnerable to cumulative stress throughout life. In contrast, match-mismatch hypothesis states that early-life environment shapes coping strategies in a manner that enables individuals to optimally face similar environments later in life, where early-life stress may not necessarily elicit adverse consequences but may be of adaptive value.

Data presented in this thesis indicate that the effects of early-life

experience may depend on inborn stress reactivity. Prolonged maternal separation (3 hrs) for first two weeks of postnatal development conferred beneficial behavioral effects in stress sensitive Wistar Kyoto (WKY) rats, but not in the stress resilient Wistar rats. Furthermore, beneficial behavioral effects were accompanied by changes in cardiovascular indices indicative of improved cardiovascular health. These behavioral and cardiovascular changes were accompanied by an increase in global DNA methylation in the hippocampus. When post-weaning WKY rats were fed with methyl-donor supplemented diet, beneficial behavioral effects were observed, reminiscent of those elicited by maternal separation. These changes were also accompanied by protective cardiovascular adaptations. Taken together, these data suggest that early-life experience has a strong effect on the behavior, brain development, and cardiovascular function throughout life. These programming effects may be mediated by epigenetic changes (i.e. differential DNA methylation) in select brain regions.

On the other hand, aggression has also been implicated in cardiovascular disorders such as coronary heart disease. Previous works has shown that rats with high levels of aggression show signs of autonomic impairment and increased susceptibility to arrhythmias. However, it is not clear whether trait aggression may be influenced by early-life experience, and whether trait aggression and early-life experience may act in concert or independently in the regulation of cardiovascular function. The current study found that trait aggression (quantified by attack behavior in the resident-intruder test) was not

impacted by the experience of maternal separation, suggesting that aggression is a stable and heritable trait that is not impacted by early-life environment in WKY rats. Trait aggressive rats showed increases in: resting blood pressure, wall-to-lumen ratio of the thoracic aorta, vascular sensitivity to phenylephrine application, and norepinephrine levels in left ventricle. In contrast, maternal separation did not impact resting blood pressure, but decreased resting heart rate and increased heart rate variability instead. These observations suggest that early-life experience and trait aggression affect distinct domains of cardiovascular function. Future studies will be required to tease out the biological mechanisms responsible for these programming effects of early-life experience and trait aggression.

DEDICATION

This dissertation is dedicated to my family members, mentors, lab members, committee members, CMBD theme, and friends. I greatly appreciate the support and patience throughout my academic career over these years. Every one of you has helped in one way or another to pursue this degree. My special dedication goes to my late uncle Ganesh Rana, who would have been proud of my achievement.

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LIST OF ABBREVIATIONS

BP	Blood Pressure
CV	Cardiovascular
DBP	Diastolic Blood Pressure
EC_{50}	Half Maximal Effective Concentration
ELE	Early Life Experience
FST	Forced Swim Test
HPA	Hypothalamic-Pituitary-Adrenal Axis
HR	Heart Rate
HRV	Heart Rate Variability
MAP	Mean Arterial Pressure
MDD	Major Depressive Disorder
MS	Maternal Separation
NA	No Aggression
NH	Neonatal Handling
OFT	Open Field Test
P	Postnatal Day
PAR	Predictive Adaptive Response

SBP	Systolic Blood Pressure
sBRS	spontaneous Baroreceptor Reflex Sensitivity
SDNN	Standard Deviation of Inter-Beat-Interval
SEM	Standard Error of the Mean
TA	Trait Aggression
WKY	Wistar-Kyoto

CHAPTER 1:

GENERAL INTRODUCTION

Major Depressive Disorder

Major Depressive Disorder (MDD) is a debilitating and multifactorial mood disorder with a high lifetime prevalence. The World Health Organization estimates that 350 million people are affected globally by this disease, and it is recognized as the leading cause of disability and global disease burden (The-World-Health-Organization, 2012). The diagnostic criteria for major depression include five physical and emotional symptoms that must be present during the same two-week period together with either depressed mood or anhedonia (i.e. loss of interest in pleasurable activities) (DSM-5, 2013). The physical symptoms of depression include alterations in sleep, appetite, and psychomotor retardation, while guilty feelings, impaired concentration, and suicidal thoughts are part of emotional symptoms. Furthermore, MDD is also with commonly associated with chronic medical conditions (Evans et al., 2005) and is often co-morbid with anxiety (Brent et al., 1998), thus making MDD a complex and multifactorial illness.

Depression and Emotional Co-morbidity

Depression if often associated with various other co-morbid psychiatric disorders making the disease itself more complex and difficult to treat. Due to the

high occurrence of comorbidity between psychiatric disorders, high interest has been directed towards comorbidity studies. For example, epidemiological studies suggest that the comorbidity rates for depression and anxiety ranges from 40%-80% across Europe and the United States of America (Kessler et al., 1994 ; de Graaf et al., 2003 ; Jacobi et al., 2004). Comorbidity of depression with other conditions worsens the prognosis, increase treatment resistance, and increases burden of disease (Lamers et al., 2011). Specific personality traits, such as social inhibition, have also been recognized to augment disease severity and treatment resistance in depressed individuals (Crawford et al., 2007).

Depression and Cardiovascular Dysfunction

In addition to neuropsychiatric co-morbidity, various medical disorders have also been associated with MDD. Some of these include neurological disorders (Parkinson's disease, Alzheimer's disease, and stroke), cancer, HIV/AIDS, and cardiovascular disorders (Krishnan et al., 2002). Clinical and epidemiological findings in humans suggest that MDD and cardiovascular disorders are highly comorbid with a bi-directional relationship (Penninx et al., 2001 ; Carney et al., 2005 ; Lichtman et al., 2009). Although 2-9% of general population suffer from depression, its prevalence in myocardial infarction survivors is estimated to be 45% (American-Psychiatric-Association, 1994). Furthermore depressed patients with no history of heart disease are also at risk for cardiovascular pathology (Grippo et al., 2002), and 50% of patients who are depressed at the time of initial diagnosis of cardiovascular disease have a prior

history of depression (Carney et al., 1987). Depression has also been found as an independent risk factor for coronary artery disease (Barefoot et al., 1996) and predisposes an individual to myocardial infarction, sudden death, thrombosis, and arrhythmias (Musselman et al., 1998). Furthermore, depressed patients display functional cardiovascular symptoms, such as increased heart rate and reduced heart rate variability, similar to those of cardiovascular diseases(Lahmeyer & Bellur, 1987 ; Krittayaphong et al., 1997). These heterogeneous brain and body alterations suggest that they result from dysregulation in multiple neural systems that control cognition, emotion, autonomic function, and motor function. Treatments for depression are not entirely efficacious, in part, because the pathobiology of the illness remains poorly understood (Pigott et al., 2010).

Baroreflex Sensitivity

Arterial baroreflex sensitivity is an important characteristic of baroreflex control for maintaining blood pressure, assessed by relating heart rate (HR) fluctuations to blood pressure fluctuations. The baroreflex is a homeostatic mechanism for maintaining blood pressure that provides a negative feedback loop to decrease blood pressure in response to elevated blood pressure and vice versa. An impaired baroreflex is associated with a number of adverse cardiovascular events (Grippo et al., 2002). Some of the early research has reported that baroreflex sensitivity is reduced in depressed cardiac patients (Watkins & Grossman, 1999). The arterial baroreflex reflects the function of sympathetic and parasympathetic nervous systems along with central nervous

system mechanisms involved in cardiovascular control (Grippo et al., 2002). These previous findings draw attention to the need of studying baroreceptor function in depression with comorbid cardiovascular dysfunction and the underlying neural mechanism.

Heart Rate Variability

Heart rate variability reflects the interaction of sympathetic and parasympathetic systems to vary the interval between consecutive heart beats. An abnormal variability in heart rate may imply an impairment of the autonomic nervous system (Rechlin et al., 1994), while its reduction indicates increased sympathetic tone, or decreased parasympathetic tone, or both. Depression is strongly correlated with decreased heart rate variability, where it is a risk factor for negative cardiovascular events and mortality in cardiac patients. Decreased heart rate variability is also observed in cardiac patients with severe coronary artery disease, heart failure, and myocardial infarction(Ryan et al., 1976 ; Karemaker & Lie, 2000), and it is a significant predictor of mortality following myocardial infarction (Kleiger et al., 1987 ; Tapanainen et al., 2002).

Depression and Stress

The etiology of depression has been thought to be precipitated by genetic and environmental factors. Stress is a state of disturbed homeostasis that is followed by physiological, behavioral, and mental adaptive response (stress response) to return to the previous homeostasis or a new homeostasis after effective adaptation (McEwen, 2000). There is a wide consensus from pre-clinical

and clinical research that stressful life events trigger depressive episodes with long term psychological impacts. However, there is not enough data to explain any specific mechanism linking stress exposure and stress response in the occurrence of depressive symptoms and depression itself (Bartolomucci & Leopardi, 2009). Although stressful life events are one of the major etiologic factors in triggering depressive episodes, the onset of depression cannot be solely explained by stressful life events only, with the key question in mood disorder research is that of the vulnerability and resilience within individuals (Feder et al., 2009). Genetic, environmental, epigenetic, and neurobiological studies have shown that resilience is mediated by adaptive changes in several neural circuits that involve various neurobiological and neurotransmitter pathways leading to adaptive coping to stress (Feder et al., 2009).

Animal Models of Depression and Anxiety

Various animal models have been utilized to understand the underlying neurobiological mechanisms of depression and anxiety. However, modeling human depression in animals has been a challenging task due to subjective nature of psychological and physiological symptoms and along with lack of specific genetic and physiologic biomarkers (Nestler & Hyman, 2010). None of the animal models of depression and anxiety that have been proposed can perfectly replicate the human disorder, but they are invaluable tools by which mechanisms underlying such disorders can be uncovered, thus eventually facilitating the development of novel therapeutic approaches (Overstreet, 2012).

The major strategies behind the development of animal models are: a) breeding rats that exhibit distinct anxiety- and/or depression- like behavior ([such as low responder rats (Stedenfeld et al., 2011)), and b) use of rat strains that show differences in anxiety/depression- like behavior. Some of the genetic rat models currently used to study depression, such as Wistar-Kyoto (WKY) and Learned Helpless rats (Overstreet, 2012), present a unique opportunity to study the depressive phenotypes and underlying neural abnormalities that develop over the lifespan. The WKY rats were originally bred parent Wistar strain as the normotensive control for the spontaneously hypertensive rat (Lerman et al., 2005). However, WKY rats became established as animal model for depression, where these rats were found to exhibit increased immobility in the forced swim test, decreased activity in the open field test, and were more susceptible to stress-induced ulcers (Pare, 1989, 1992, 1993, 1994).

Apart from genetic models, there are several environmental models to study the etiology of mood disorders. Some of the examples of these environmental manipulation models include: maternal separation (MS), chronic mild stress, social defeat, repeated restraint stress, and learned helplessness (see (Menard et al., 2015) for review). Although, none of the mentioned environmental models fully recapitulate human depression, each of these models has their own advantages and disadvantages that capture different aspects relating to human condition (Menard et al., 2015).

Early-life Stress – Adaptation vs. Maladaptation

In the field of stress biology, most research focuses on the stress-induced pathologies. However, there is a relative paucity of work on stress-induced adaptation. For instance, although every individual experience stressful life events, relatively few people are prone to stress-induced psychiatric disorders while the rest are resilient (Feder et al., 2009). In fact, the adaptation to stress may confer beneficial effects later in life. Thus, several hypotheses have been proposed to explain the effects of early-life stress on health in adulthood. These hypotheses include: a) cumulative or multiple hit hypothesis, b) stress inoculation hypothesis, and c) match/mismatch hypothesis.

Cumulative or Multiple Hit Hypothesis

"Cumulative stress" hypothesis or "multiple hit" hypothesis states that stress during early life predisposes individuals to stress later in life (Nederhof & Schmidt, 2012). Extensive evidence supports the view of the negative impact of early-life stress, and the general belief of worsening outcomes following increasing stress exposure (Taylor, 2010). It is thought that the cumulative effect of recurring stress exposure (during early life and in adulthood), in conjunction with genetic risks, leads to an increased risk for the development of mental and physical disorders (McEwen, 2006).

Stress Inoculation Hypothesis

Stress Inoculation hypothesis states that brief intermittent stress exposure early in life induces the development of subsequent stress resistance later in life

(Parker et al., 2006). It is based on the notion that mild stress that requires brief HPA-axis activation is important for subsequent stress resistance (Parker et al., 2006). It is proposed that mild stress during early life may “inoculate” the developing organism exacerbating the stress resistance. Repeated stress exposure during childhood period in non-human primates has been shown to confer protective neuroendocrine effects in adulthood, supporting the notion of the beneficial stress inoculation hypothesis (Parker et al., 2005 ; Lyons & Parker, 2007).

Match-Mismatch Hypothesis

Harmful effects of early-life stress on health have been well recognized. However, emerging evidence on the effects of early-life stress suggests a more nuanced view where the outcome may not be always adverse. For example, the match/mismatch hypothesis of disease states that stress during early life can have adaptive value to an individual that faces later stressful environments or experiences during adulthood (Santarelli et al., 2014). For example, human and rodent offspring exposed to malnutrition stress *in utero* develop metabolic dysfunction, only when raised under nutrition-rich environment (environment mismatch between early and later life), but not under nutrition-scarce conditions (environment match between early life and later life) (Ravelli et al., 1998). Similarly, this concept can be extended to mood disorder research also (Schmidt, 2011). The concept of predictive adaptive response (PAR), which means that an individual will use experience of past stressors to augment coping with future stressors also, supports the idea of adaptability to early stressors (Gluckman et

al., 2007). For example, rat pups exposed to the stress of either poor maternal care or 24-hour MS exhibited increased memory performance and long-term potentiation as adults when tested under stressful conditions (matched) but not under non-stressful conditions (mismatched) (Oomen et al., 2010). This PAR is thought to be strongest in stress susceptible individuals and may be evolutionarily conserved by rapidly changing environmental conditions across generations (Gluckman et al., 2007).

Maternal Separation: Modeling Early Life Stress

Early-life stress has life-long impact on brain, behavior, physiology, and stress reactivity. Traumatic events during early life contribute to the development of individual differences in the ability to cope with future stressful events during adulthood (Menard et al., 2015). To model early-life stress in rodents, MS paradigm was originally developed by Plotsky and Meaney (Plotsky & Meaney, 1993) and has since been widely used . In this model, rat pups are separated from their dams from 3 – 24 hrs over a period ranging from one 24 hr separation to daily separations on 14 consecutive days. The comparison control also varies between 15 minute daily separations (termed neonatal handling (NH)), or no intervention/handling at all. Although, MS was originally thought to be detrimental to behavior and physiologic function during adulthood, discrepancies occur in MS literature with certain studies unable to confirm its negative effects. These include studies that fail to replicate MS-induced increases in anxiety- and/or depressive- like behavior (Roman et al., 2006 ; Hulshof et al., 2011 ; Nam

et al., 2014a), and increased fear conditioning (Chocyk et al., 2014). These conflicting results likely stem from a host of factors, including varied experimental procedures, gender, and rat strain (Lehmann & Feldon, 2000). Importantly, these findings suggest that early-life stress is not uniformly deleterious and its long term effects may depend upon an organism's innate level of stress reactivity (Neumann et al., 2006).

Early-life Stress and Cardiovascular Function

Epidemiological studies suggest life-long impact of early life environment and experience on cardiovascular function. Longitudinal study on former war evacuees found that children that were war-evacuated have higher cardiovascular morbidity along with higher prevalence of type 2 diabetes and hypertension (Alastalo et al., 2009). Another large-cohort retrospective study reported that ischemic heart disease was found to be mediated by psychological risk factors that were further associated with adverse childhood experience (Dong et al., 2004). Although numerous clinical and epidemiological studies report the association between early-life stress and cardiovascular diseases, only a few studies investigated the effect of early-life stress on cardiovascular function in pre-clinical models. Repeated MS in rodents has been found to sensitize rats to angiotensin-induced hypertension, and vascular inflammation in adult life (Loria et al., 2010b). Furthermore, early-life stress was shown to downregulate endothelin receptor expression, which was accompanied by enhanced pressor response to acute stress during adulthood (Loria et al., 2010a). Another study

from a different group found that maternal separation had mild alteration in cardiac autonomic tone and heart structure when rats were further exposed to stressor during adulthood (Trombini et al., 2012). Epigenetic mechanisms, such as DNA methylation and histone modification, have been suspected to mediate these effects of early-life stress (Loria et al., 2014).

Personality Traits and Cardiovascular Function

Personality, or distinct dimensions of emotionality, has also been linked to cardiovascular disease (Kupper & Denollet, 2007). Pioneering work by Friedman and Rosenman identified that individuals with a certain behavioral profile (termed Type A) have an increased risk of heart disease (Friedman & Rosenman, 1959). Recently, the concept of the Type A behavioral profile has been more refined to investigate the association of personality traits with heart disease. Type A individuals with negative affectivity and social inhibition are deemed to be more vulnerable to cardiovascular dysfunction (Denollet et al., 1996 ; Denollet, 2000). A new type of personality trait has been proposed that includes Type A characteristics with negative affect and social inhibition termed Type D (for "distressed") personality profile of individuals who are likely to experience emotional and interpersonal difficulties along with poor health (Kupper & Denollet, 2007). Type D individuals experience more anger, manifest more hostility and physical aggression, and express more anger toward others (Kupper & Denollet, 2007).

Early-life Stress and Personality Traits

Reports on the effects of early-life stress on personality characteristics suggest a complex relationship with species-specific and age-dependent effects. In humans, adoptees from Romanian orphanages seem to display significantly more indiscriminately friendly behavior toward new adults following (Chisholm, 1998). In rodents, one study reports higher aggressiveness to an intruder by MS exposed rats (Veenema et al., 2006). In contrast, MS exposure was shown to suppress adult inter-male aggression in C57BL/6 mice despite MS-induced increase in anxiety and depression like behavior (Tsuda et al., 2011). It has been suggested that the development of psychopathological and aggressive behaviors is due to early environment in conjunction with genetic predisposition (Haller et al., 2014). The link between early-life stress and aggression is not fully understood with inconsistencies in the results requiring further investigation.

Epigenetic Mechanisms

Early postnatal development is the time when environmental influences can exert long-lasting changes in brain plasticity (Roth & Sweatt, 2011). Early environment shapes the organization of neural circuits and is thought to be the basis for either vulnerability or resilience to stress (Roth & Sweatt, 2011). Epigenetic mechanisms are thought to mediate these effects and contribute to the gene-environment interaction throughout the lifespan. Epigenetic mechanisms include regulation of gene expression via histone modifications, DNA methylation and non-coding RNAs without changing the DNA sequence

(Loscalzo & Handy, 2014). Growing evidence suggests that these epigenetic mechanisms contribute to early-life induced programming of behavioral and physiological functions in adulthood (Champagne & Meaney, 2001 ; Loria et al., 2014 ; Loscalzo & Handy, 2014).

DNA Methylation

DNA methylation involves covalent addition of methyl groups to the C5 position of cytosine, catalyzed by DNA methyltransferases (DNMTs) enzymatic activity. Repressor proteins, including the methyl-binding domain protein MeCP2 and the histone deacetylases HDACs bind to the methylated cytosines, often resulting in the suppression of gene transcription (Bird, 2002 ; Miranda & Jones, 2007). Although DNA methylation is generally believed to have gene silencing function, recent studies suggest that the methyl-binding domain protein MeCP2 is also associated with promoting gene expression (Chahrour et al., 2008 ; Cohen et al., 2008). In rodents, it has been shown that early-life experience (mediated through maternal behavior) mediates epigenetic profile in the offspring through DNA methylation, especially in the stress response system (Meaney & Szyf, 2005 ; Szyf et al., 2005 ; Zhang et al., 2010). In humans, increased DNA methylation of glucocorticoid response elements of the FK506 binding protein 5 (FKBP5) gene is associated with childhood trauma (Klengel et al., 2013). DNA methylation changes are also heritable, thus being an ideal substrate to investigate the long lasting epigenetic alteration to postnatal environment (Roth & Sweatt, 2011).

Specific Aims

1) Determine the behavioral effects of MS in rats with and without heightened susceptibility to stress.

2) Determine the effects of early-life experience differences on trait aggression, and the impact of these factors on baseline cardiovascular factors.

3) Determine effects of diet based methyl donor supplementation vs. methyl donor depletion on depression- and anxiety- like behavior and cardiovascular function.

INBORN STRESS REACTIVITY SHAPES ADULT BEHAVIORAL CONSEQUENCES OF EARLY-LIFE MATERNAL SEPARATION STRESS

by

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CHAPTER 2: INBORN STRESS REACTIVITY SHAPES ADULT BEHAVIORAL CONSEQUENCES OF EARLY-LIFE MATERNAL SEPARATION STRESS

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2.1 INTRODUCTION

Adverse life experiences during the early developmental period have long-term effects on the brain, stress-elicited behaviors, endocrine function, and physiological responses in a variety of species (Talge et al., 2007). Early-life adversity in humans can predispose individuals to neuropsychiatric disorders and suicidality (Middlebrooks & Audage, 2008). Childhood abuse and neglect are also associated with adverse behavioral risk factors in adulthood, including smoking, physical inactivity, obesity, depression, and cardiovascular disease (Dong et al., 2004).

Prolonged early-life maternal separation (MS) is a widely used rodent model of early-life adversity where pups are deprived of maternal contact for variable time periods during the early weeks of life (Lehmann & Feldon, 2000). Most reports document myriad negative effects of MS including: increased anxiety-like behavior (Lippmann et al., 2007), depression-like behavior (Gardner et al., 2005), and exaggerated hypothalamic-pituitary adrenal (HPA) axis stress responses (Plotsky & Meaney, 1993). There are discrepancies in the MS literature, with certain studies unable to confirm such behavioral and

neuroendocrine findings. For instance, some reports failed to see effects of MS on anxiety measures (Roman et al., 2006 ; Hulshof et al., 2011), and another showed that MS decreased contextual and auditory fear conditioning rather than increasing it (Chocyk et al., 2014). These conflicting results likely stem from a host of factors, including varied experimental procedures, gender, and rat strain that was used (Lehmann & Feldon, 2000). Importantly, these findings suggest that early-life stress is not uniformly deleterious and its long term effects may depend upon an organism's innate level of stress reactivity (Neumann et al., 2006).

The current study tested the hypothesis that early-life MS elicits disparate effects on rat strains that exhibit innate differences in stress susceptibility. To do so, we applied the early-life MS paradigm to two rat strains: Wistar-Kyoto (WKY) rats, a well-established model animal of heightened depressive-/anxiety-like behavior and stress vulnerability (Nam et al., 2014a), and genetically similar Wistar rats. WKY rats manifest heightened endocrine and physiological responses to stress (Pardon et al., 2002), increased stress-induced ulcers and gastrointestinal dysfunction (Pare, 1989), and display robust behavioral despair and learned helplessness, a core feature of major depression in humans (Pare, 1989). Given WKY rats' high baseline levels of anxiety/depression-like behavior and stress susceptibility, we initially hypothesized that they would be particularly vulnerable to the deleterious effects of early-life MS. On the other hand, Wistar rats typically display low levels of basal anxiety-/depressive-like behavior relative to WKY rats (Nam et al., 2014a). Our data indicate that MS elicits disparate

effects on Wistar vs. WKY rats. Consistent with previous studies, early-life MS led to increased anxiety-like behavior in the Wistar offspring. However, MS elicited protective effects in the WKY offspring, improving their anxiety- and depression- like behaviors along with social behaviors.

2.2 METHODS

All animal handling and experimental procedures were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee and conformed to the Guide for the Care and Use of Laboratory Animals.

2.2.1 Animals and Early-Life Manipulation

WKY and Wistar female and male rats (n=8/sex/strain) were purchased from Charles River Laboratories (Kingston, NY) and housed in a temperature-controlled facility (kept at 21-23 °C, 50-55% humidity) with a 12/12 h light-dark cycle (lights on at 6:00 a.m.). Male/female pairs were mated for 14 days, and at birth (P0) litters were randomly assigned to one of two groups: (a) neonatal handling group or (b) MS group (n = 4 litters/group/strain). Litters assigned to the MS group and neonatal handling group were separated from their dam daily for 180 min and 15 min, respectively, between 8:30 a.m. – 12:00 p.m. from P1-P14 as previously described (Clinton et al., 2014). Dams remained in the home cages while separated litters were transferred to a different room in a small cage placed on a heating pad (~37°C). Littermates remained in close contact throughout the

separation period and were returned to home cage after the conclusion of the 15- or 180-min period.

After the final separation on P14, litters remained undisturbed until weaning on P21. Only male pups were chosen for subsequent behavioral tests performed in adulthood. Rats of the same strain and same early-life experience (neonatal handling or MS) were housed together 3 per cage. Animals were left undisturbed except for weekly weighing and standard cage changes from weaning until P60+ when behavioral testing commenced.

2.2.2 Behavior Test Battery

Behavior tests were conducted under dim light conditions (30 lux) between 8:30-11:30 a.m.. Animals were placed in the testing room overnight for habituation before all tests except for forced swim test (FST). The following tests were conducted (in the order indicated): open field test (OFT), social interaction, and FST.

OFT and FST were conducted as previously described (Nam et al., 2014a). Social interaction testing was conducted in a rectangular black Plexiglas box (91 × 61×30 cm) with a black floor, which was divided into three chambers (zones) separated by two black Plexiglas dividers with openings in the center to allow animals to move freely between zones. Testing was conducted over two days (10 min/day). On Day 1, the test rat was placed in the neutral zone (middle chamber), while one of the other zones contained an empty cylindrical metal bar-cage in a corner of the zone. The third zone contained a male stimulus rat within

an interaction cage placed in a corner. The metal bars of the interaction cage allowed rats to interact, but prevented any aggressive encounters between animals. On Day 2, the test rat was again placed in the neutral zone; one of the other zones contained a male stimulus rat within the interaction cage and the third zone contained a female stimulus rat within its interaction cage. Position of the male stimulus rat was switched between test days to eliminate side preference. Stimulus rats were age-matched and of the same strain as the test animals, and were previously habituated to interaction cages. An approximately 2-cm wide zone around each interaction cage was designated as the interaction zone. Behavior in all tests was recorded with a digital camera, and quantified utilizing Ethovision® XT 8.0 software (Noldus, Wageningen, The Netherlands).

2.2.3 Statistical Analysis

Data from the OFT, Social Interaction test Day 1, and FST were analyzed via two-way ANOVA, with strain and early-life treatment (MS/neonatal handling) as independent variables. When necessary, post-hoc analysis was performed using independent samples t-test within each strain independently and p-values were adjusted using Holm-Bonferroni correction. Data from Social Interaction Day 2 were analyzed separately for the two rat strains. Within each strain, sex of stimulus rat and early-life manipulation were used as independent variables with Bonferroni tests post-hoc. Statistical analysis was performed using GraphPad Prism 6.0. Significance was set at $p < 0.05$; results are presented as mean ± SEM.

2.3. RESULTS

Both WKY and Wistar offspring gained weight from weaning through adulthood and there was no effect of early life experience (MS or neonatal handling) on their weight gain (data not shown).

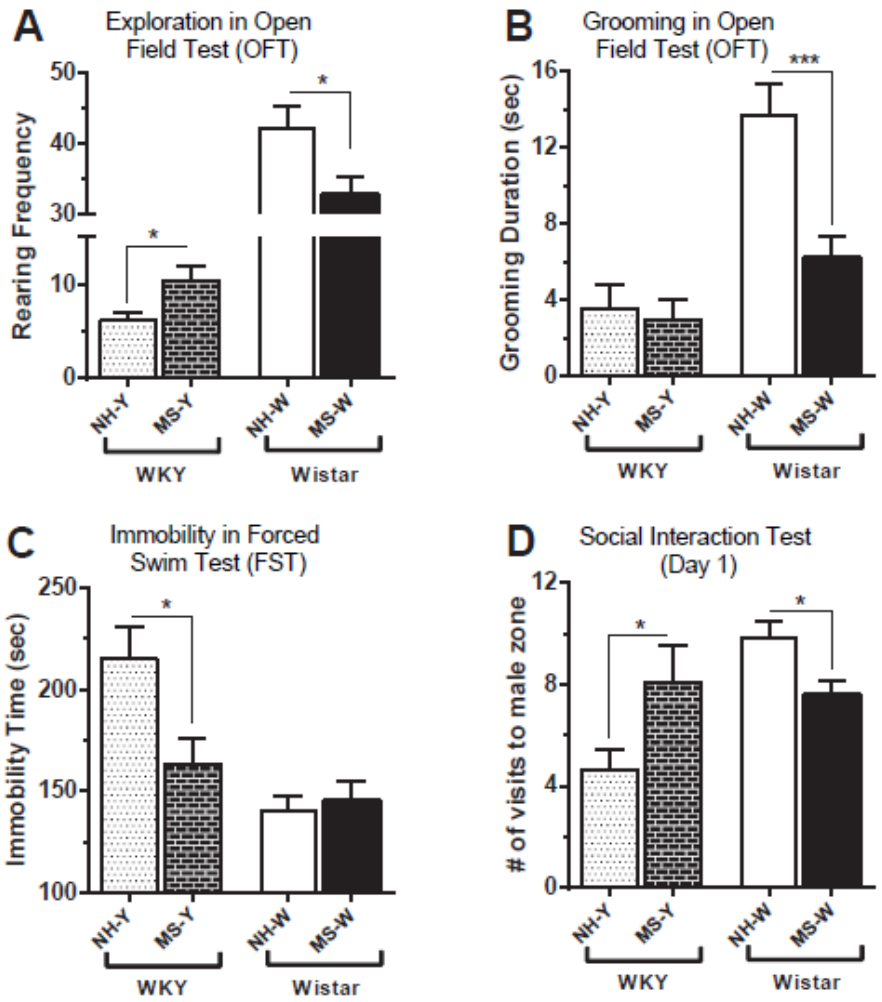

Figure 2.1. Contrasting effects of early-life experience on WKY vs. Wistar rats. Two-way ANOVA revealed significant early-life treatment x strain interactions for: rearing frequency (A) and grooming in OFT (B); immobility in FST (C); frequency to male zone on social interaction testing (D). Abbreviations: NH-Y – neonatally-handled WKYs, MS-Y – maternally-separated WKYs, NH-W – neonatally-handled Wistars, MS-W – maternally-separated Wistars. * – $p < 0.05$, ** – $p < 0.01$, *** – $p < 0.001$.

2.3.1 Contrasting effects of MS

MS differentially affected OFT behavior in WKY vs. Wistar offspring. First, there was a significant main effect of strain ($F_{(1, 61)} = 106.4$, $p < 0.0001$) with Wistar rats generally displaying greater amounts of rearing compared to WKYs (Fig. 1A). Interestingly, there was a significant strain x early-life treatment interaction ($F_{(1, 61)} = 5.728$, $p < 0.05$). Post-hoc analysis showed that MS elicited disparate effects within each strain, with MS-exposed WKY rats showing increased rearing in the OFT compared to the neonatal handing-exposed WKY offspring ($p < 0.05$). On the other hand, Wistar offspring that were exposed to MS displayed decreased rearing compared to Wistars that been exposed to neonatal handling ($p < 0.05$; Fig. 1A). Similar to rearing, grooming duration in the OFT was also differentially impacted by MS, with significant effects of: strain ($F_{(1, 62)} = 21.67$, $p < 0.0001$), early-life treatment ($F_{(1, 62)} = 7.907$, $p < 0.01$), and strain x early-life treatment interaction ($F_{(1, 62)} = 5.774$, $p < 0.05$). Post-hoc testing revealed a large decrease in the amount spent grooming in the MS-exposed Wistar rats ($p < 0.001$), but no change in the WKY rats, so that MS-exposed WKY and Wistar rats exhibited similar levels of grooming (Fig. 1B). MS also differentially impacted FST immobility in WKY versus Wistar offspring. First, there were significant main effects of strain ($F_{(1, 62)} = 17.05$, $p < 0.001$) and MS ($F_{(1, 62)} = 4.171$, $p < 0.05$), with WKY rats generally exhibiting greater FST immobility compared to Wistar rats (Fig. 1C). There was also a significant strain x early-life treatment interaction ($F_{(1, 62)} = 6.240$, $p < 0.05$). Post-hoc analysis revealed that MS lead to reduced FST immobility selectively within WKY rats ($p < 0.05$), while

Wistar rats' FST behavior was unaffected by MS exposure (Fig. 1C). MS exposure also elicited different effects in WKY and Wistar offspring during Day 1 of the Social Interaction test. There was a main effect of strain ($F_{(1, 62)} = 8.105$, $p < 0.01$), but not of MS, on the frequency to visit the novel male social interaction zone on the first social interaction test day, with Wistar rats generally making more visits to the novel male compared to WKY rats (Fig. 1D). However, there was also a significant interaction of strain and early-life treatment ($F_{(1, 62)} = 12.04$, $p = 0.0010$), and post-hoc analysis revealed disparate effects of MS on WKY and Wistar offspring's social behavior. MS-exposed WKY offspring showed more visits to the novel male compared to neonatal handling-exposed WKYs. MS had the opposite effect on Wistar offspring, with MS-exposed Wistars showing less social interaction with the novel male rat relative to Wistar offspring that had been exposed to neonatal handling ($p < 0.05$ for each comparison; Fig. 1D). There were no significant main effects of strain or early-life treatment and no strain x early-life treatment interaction on the rats' interaction with an inanimate novel object (a control used in the Social Interaction Day 1 test; data not shown).

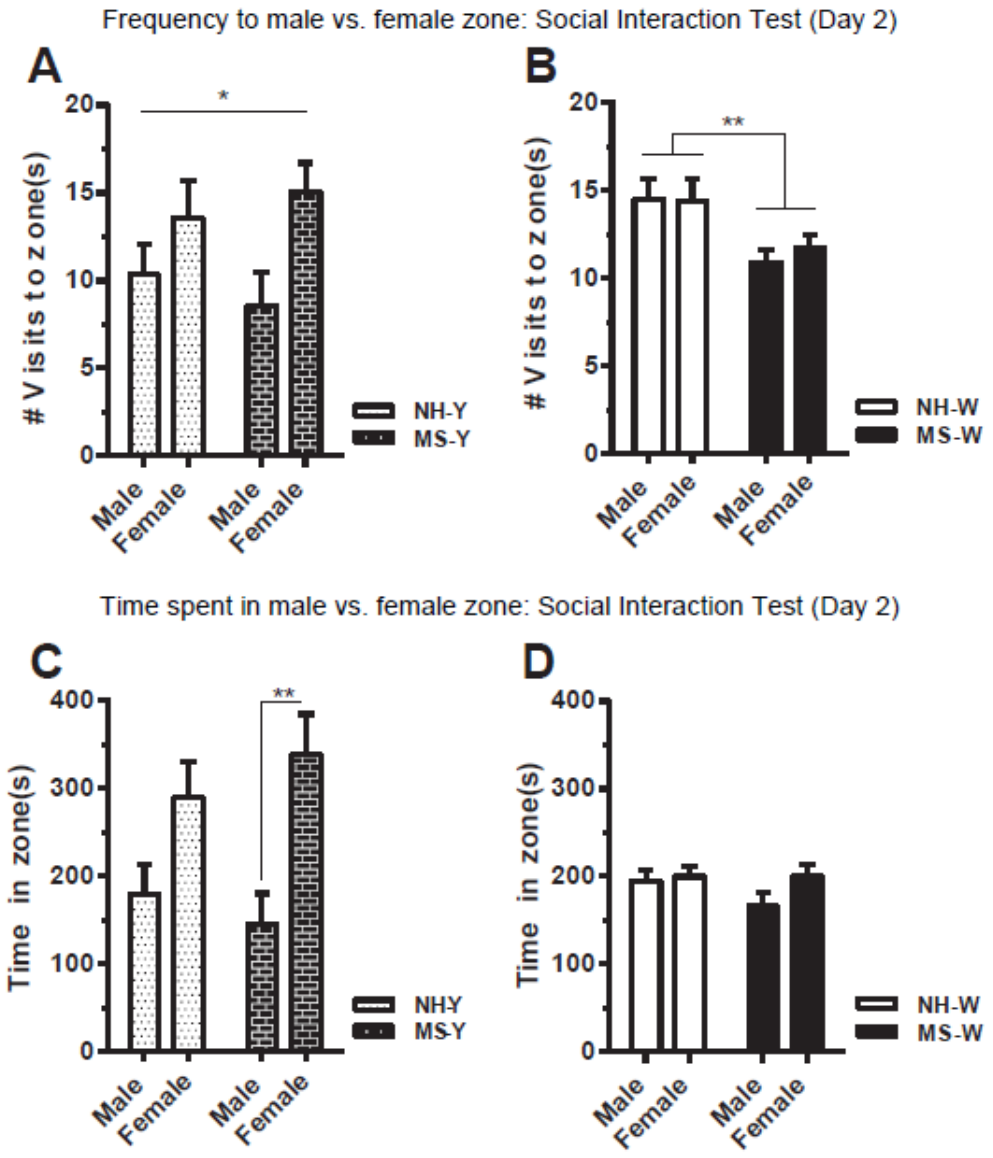

Figure 2.2. When given a choice between exploring a novel male or female on Day 2 of the social interaction test, significant main effect of sex of the stimulus rat was observed in WKY offspring (A). Wistars exhibited significant effect of early-life treatment, where maternally-separated rats made fewer visits to both male and female zones (B). Maternally-separated WKY rats spent more time interacting with the female stimulus rat (C). No differences in interaction times were observed in Wistar rats (D). Abbreviations as in Fig. 1. * – $p < 0.05$, ** – $p < 0.01$.

2.3.2 MS and male vs. female social interaction

On Day 2 of social interaction testing, rats were given a choice between exploring a novel male or a novel female stimulus rat. WKY groups (MS- and neonatal handling-exposed offspring) showed a preference for visiting the female stimulus rat, with more frequent visits to the female interaction zone versus the male interaction zone (main effect of sex of stimulus rat: $F_{(1,44)} = 6.853$, $p < 0.05$; Fig. 2A). There was no main effect of early-life treatment on this measure in WKY offspring, and no stimulus rat x early-life treatment interaction. We observed a different pattern of social behavior in the Wistar rats. MS-exposed Wistar offspring made fewer visits to both male and female interaction zones relative to neonatal handling-exposed Wistar offspring (main effect of MS: $F_{(1,80)} = 10.22$, $p < 0.01$) and did not show a preference for visiting female over male stimulus rats (no main effect of stimulus rat and no interaction; Fig. 1B). We also examined the effect of stimulus rat and MS exposure on the amount of time WKY and Wistar rats spent in the male/female rat interaction zones. For WKY rats, there was a significant main effect of stimulus rat, with all WKY offspring spending more time in the female interaction zone ($F_{(1, 44)} = 15.05$, $p < 0.001$). Post-hoc analysis revealed that this difference was significant in the maternally-separated, but not neonatally-handled, animals ($p < 0.01$; Fig. 2C). There was no main effect of early-life treatment or sex of the stimulus rat (and no interaction) for time spent in the male and female interaction zones was detected for Wistar rats (Fig. 2D).

2.4 DISCUSSION

The present study demonstrates contrasting effects of early-life MS in two strains of rats that exhibit innate differences in emotional behavior and stress susceptibility. In Wistar rats, MS increased anxiety-like behaviors in the OFT and decreased social behavior, which is consistent with several prior studies documenting adverse effects of MS. We were surprised to find that MS improved anxiety- and depression-like behavior in WKY offspring, leading to increased exploratory behavior (OFT), increased social interaction, and diminished FST immobility. We also observed a decrease in grooming in the Wistar, but not WKY, rats exposed to MS, which may be an indication of diminished novelty-induced exploration and increased anxiety-like behavior (van Erp et al., 1994 ; McGregor et al., 2004). These findings demonstrate contrasting effects of early life stress on adult behavior in rat strains with divergent endogenous stress susceptibility, an effect that has not been reported previously.

Although most work on early-life stress focuses on its harmful effects on current and future health, emerging evidence suggests a more nuanced view. For example, the match/mismatch hypothesis of disease posits that stress during early life is not necessarily pathological but can instead have adaptive value to an individual that faces later stressful environments or experiences during adulthood (Santarelli et al., 2014). Examples from metabolic research report that human and rodent offspring exposed to malnutrition stress *in utero* develop metabolic dysfunction when raised under non-stressful food abundant conditions, but not under food scarce conditions (Ravelli et al., 1998). The notion of

beneficial stress inoculation has been suggested based on the observation that repeated stress exposure in childhood confers protective neuroendocrine effects in adulthood (Lyons & Parker, 2007). Consistent with this idea is the concept of the predictive adaptive response (PAR), which means that an individual will use experience of past stressors to augment coping with future stressors (Gluckman et al., 2007). This idea is supported by data demonstrating that rat pups exposed to the stress of either poor maternal care or 24-hour maternal deprivation exhibited increased memory performance and long-term potentiation as adults when tested under stressful conditions but not under non-stressful conditions (Oomen et al., 2010). This PAR is thought to be strongest in stress susceptible individuals and may be evolutionarily conserved by rapidly changing environmental conditions across generations (Gluckman et al., 2007). Our present data are consistent with this theory, showing that early-life MS has positive effects in the stress susceptible WKY rats.

Previous MS studies have utilized a variety of manipulations and comparison groups to ascertain effects of early life stress, including separation paradigms of single 24-hour separation, as well as repeated daily separations lasting 1-6 hours over the first 2-3 postnatal weeks. A variety of comparison groups have been used which include groups that were: 1) non-handled; 2) animal facility reared; or 3) brief handling (3-15 min), during the early postnatal period. In the wild, the dam must leave the nest on a regular basis to search for food, and inducing brief separations via daily neonatal handling is one way to mimic this effect. Extensive literature demonstrates the contrasting effects of

neonatal handling vs. MS in terms of HPA axis reactivity and stress-elicited behaviors in adulthood (Plotsky & Meaney, 1993), thus we decided to use these manipulations in the present study.

It's important to keep in mind that the effects of MS depend on the strain and genetic endowment of rats experiencing it. In our previous study we evaluated the effects of MS on behavior and endocrine function in the selectively-bred Low (bLR) and High (bHR) responder rats (Clinton et al., 2014). These rats were bred from the Sprague-Dawley strain and show striking differences in their depressive- and anxiety-like behavior, with bLRs showing a profile similar to the WKY rats and bHRs resembling Wistar rats (Flagel et al., 2014). While MS did not induce changes in the bLR/bHR behaviors on the OFT and FST, their endocrine responses were differentially impacted with the bLRs manifesting an augmentation of adrenocorticotropic hormone secretion in response to an anxiogenic stimulus (i.e. exposure to the light/dark box) and bHRs showing its blunting (Clinton et al., 2014). While beyond the scope of the current study, it will be important to determine the impact of MS on the HPA axis activity in the WKY and Wistar rats given that the activity of this system has the potential to shape to depression and anxiety (Kathol et al., 1989). Stress exposure, such as MS, leads to increased secretion of corticosterone, which has strong effects on brain development (Catalani et al., 2000). MS also leads to increases in mineralocorticoid and glucocorticoid receptors in the hippocampus, which mediate corticosterone signaling in the brain (Ladd et al., 2004). It is therefore likely that this system mediates the observed behavioral effects in our study. It is

also important to note that MS may be a relatively mild stressor, and that more severe stressors, such as more prolonged maternal separation or chronic stress exposure, may have different consequences in both strains and may be detrimental to their development and behavior.

Previous studies reported decreased weight in maternally-separated rats before and after weaning, suggesting that the separation suppressed maternal milk availability or circulating growth hormone levels (Vallee et al., 1996). In the current study, we did not see an effect on Wistar or WKY offspring's physical development, suggesting the observed behavioral differences are not due to metabolic differences induced by MS. Instead, the contrasting effects of neonatal handling and MS on WKY vs. Wistar offspring may be mediated by alterations in maternal care induced by the manipulations. Variations in maternal care significantly impact stress-elicited behavioral and endocrine responses in adulthood, and contribute to the effects of brief early-life separation (as in the neonatal handling paradigm) (Liu et al., 1997). These observations indicate a key role of maternal behavior in shaping the effects of early-life manipulations on adult offspring's behavior. A limitation of the present experiment is that we did not compare maternal behaviors under MS and neonatal handling; future studies will be required to address this.

In previous study we failed to see an effect of MS or neonatal handling on depressive-/anxiety- like behaviors in WKY offspring (Nam et al., 2014a). However, in that study, pregnant WKY females were purchased and delivered to our animal housing facility around gestational day 15. Given the heightened

sensitivity of WKY rats to stress and other environmental perturbations, it is very likely that the stress of transport significantly impacted offspring via prenatal stress and/or possible changes in maternal behavior, which could have interfered with effects of neonatal handling/MS. To circumvent this issue in the current study, we mated male/female WKY and Wistar rats in our own animal housing facility, thus eliminating the potential confound of prenatal stress. The current observations taken together with our previous report (Nam et al., 2014a) suggest significant differences in the behavioral profiles of WKY rats that were: 1) reared under commercial conditions and shipped as adults; 2) shipped *in utero*; and 3) reared in our animal facility from conception through adulthood. These observations highlight the importance of minimizing exposure to external stressors throughout the lifespan of WKY rats, including during the prenatal period, in studies examining their behavioral alterations.

In conclusion, our current data suggest that the effects of early-life MS on anxiety- and depression-like behavior in adulthood may depend on innate stress reactivity. Future studies will be required to determine the role of maternal care and the neurobiological mechanisms that mediate these effects.

CHAPTER 3: INDEPENDENT EFFECTS OF EARLY-LIFE EXPERIENCE AND TRAIT AGGRESSION ON BASELINE CARDIOVASCULAR FUNCTION

3.1 INTRODUCTION

Extensive evidence has implicated depression, anxiety, and chronic life stress as predisposing factors for the incidence and progression of heart disease (Musselman et al., 1998 ; Rozanski et al., 1999 ; Barth et al., 2004). Similarly, distinct dimensions of emotionality have also been linked to cardiovascular disease (Kupper & Denollet, 2007). This notion was first raised by Friedman and Rosenman who reported a cluster of personality traits, termed Type A behavior pattern, that increased the risk of heart disease (Friedman & Rosenman, 1959). Such individuals tended to be highly competitive and interacted with others in an aggressive or hostile manner. In contrast, individuals with the Type B behavioral profile tended to be more even tempered and patient, and were not at an increased risk for heart disease (Friedman & Rosenman, 1971). Since then the concept of the Type A behavioral profile has been refined, and more recent work has demonstrated that specific behavioral traits likely mediate much of the association between Type A profile and heart disease, namely negative affectivity and social inhibition (Denollet et al., 1996 ; Denollet, 2000). The constellation of these two traits has been proposed to form Type D (for "distressed") personality profile of individuals who are likely to experience emotional and interpersonal difficulties along with poor health (Kupper &

Denollet, 2007). Type D individuals experience more anger, manifest more hostility and physical aggression, and express more anger toward others (Kupper & Denollet, 2007). In the general population 13-25% of individuals can be classified as Type D, while 26-53% of cardiac patients exhibit Type D characteristics (Kupper & Denollet, 2007). Both negative affectivity and social inhibition, the two components of Type D personality, appear to be highly heritable and stable over time (Kupper et al., 2007), though they may be modifiable by distinct environmental factors (Martens et al., 2007 ; Kupper et al., 2011).

In the animal kingdom, aggressive behaviors are evolutionary traits that are required for survival in the face of competition from rivals over territory, food, and reproductive opportunities (i.e. competition for mates) along with the necessity for self-preservation when faced with predator threat (Natarajan & Caramaschi, 2010 ; Lindenfors & Tullberg, 2011). However, excessive aggression can be pathological and is associated with a host of emotional and cardiovascular abnormalities. Specifically, highly aggressive mice that manifest short attack latencies in social encounters exhibit numerous autonomic, endocrine, and neurobiological alterations (Natarajan & Caramaschi, 2010). These include lower resting heart rate, bunted sress-evoked glucocorticoid secretion, and decreased brain serotonin levels accompanied by high 5-HT1A autoreceptor activity and low serotonin reuptake transporter activity (Veenema et al., 2003 ; Caramaschi et al., 2007, 2008). Work in selectively-bred aggressive Groningen rats, which manifest very short attack latencies and repeated attack

bouts in the resident-intruder test, has shown a decrease in heart rate variability, impairment of the vagal control of heart rate, and a lowered threshold for ventricular tachyarrhytmias induced by beta-adrenergic receptor stimulation (Carnevali et al., 2013). In addition wild-type aggressive rats show increased sympathoadrenal activation both at rest and in response to a social challenge (Sgoifo et al., 1998 ; Sgoifo et al., 2005). These rats are also characterized by sympathetic predominance in the cardiac autonomic control, and they have greater susceptibility to cardiac arrhythmias following social defeat (Sgoifo et al., 1998 ; Sgoifo et al., 2005). These findings in rodents are reminiscent of those in humans that have demonstrated a strong relationship between aggression and hostility and the increased risk for cardiac disease (Smith et al., 2004).

Early-life experience (ELE) can have a strong influence on life-long health and disease. Numerous studies provide evidence that adverse life experiences during the early developmental period have long-term negative effects on the brain, stress-elicited behaviors, endocrine function, and physiological responses in a variety of species (Suomi, 1991 ; Daniels et al., 2004 ; Talge et al., 2007 ; Rensel et al., 2010 ; Thompson, 2012). While these data provide extensive evidence for the role of early-life stress in eliciting maladaptive changes in adulthood, it is also feasible that stress exposure during early development elicits adaptive changes in later life. Consistent with this notion is the finding that individuals who coped with traumatic events during childhood are better able to cope with spousal loss, illness, and major accidents in adulthood as compared to those who did not experience similar stressors as children (Forest, 1990 ;

Khoshaba & Maddi, 1999). These observations suggest that stress-induced adaptations during childhood can serve a protective role later in life, in this way conferring stress-resiliency (Lyons & Parker, 2007). It is evident that adverse early environment does not always result in maladaptation resulting in pathological outcomes. In fact, the resulting effect of adverse early environment may be maladaptive or adaptive depending on the interactions among a host of variables, including: genetic, environmental, epigenetic, and social factors.

Various studies have investigated the role of ELE on social and aggressive behaviors. Previous results suggest that maternal separation (MS) exposure during early development, an established model of early-life stress (Millstein & Holmes, 2007 ; Nishi et al., 2014), leads to increased aggressive play-fighting in adolescent Wistar rats (Veenema & Neumann, 2009) and greater aggressive displays toward novel conspecifics (Veenema et al., 2006). In contrast, when Long Evans rats were tested for juvenile social behavior, no differences in attack behavior between MS-exposed rats and controls were observed (Zimmerberg & Sageser, 2011). Similarly, MS exposure was shown to suppress adult intermale aggression in C57BL/6 mice despite MS-induced increase in anxiety- and depression- like behavior (Tsuda et al., 2011).

However, it is not clear whether ELE may also alter specific emotionality domains, such as aggression, that correlate with altered cardiovascular function and which confer heightened risk of heart disease. In this study we utilized Wistar-Kyoto (WKY) rats, which have high levels of inborn anxiety- and depressive- like behavior that are proxy measures for negative affect in humans.

In our recent study we showed that in addition to their high levels of anxiety- and depressive-like behavior, WKY rats also manifest heightened social inhibition and withdrawal relative to several other comparison strains (Nam et al., 2014a). We subsequently demonstrated that ELE can significantly impact their behavior, so that prolonged MS induces adaptive behavioral changes in these rats and diminishes their anxiety- and depressive- like behaviors, and increases their social behaviors (Rana et al., 2015). In contrast, MS in Wistar rats, which are non-stress reactive, increases their anxiety-like behavior and inhibits their social interaction (Rana et al., 2015). In the present study, we extend these previous observations to determine the impact of MS on trait aggression (TA) along with cardiovascular structure and function. We demonstrate that: 1) TA is independent of MS exposure in WKY rats; 2) MS exposure correlates with lower resting heart rate (HR), increased HR variability (HRV), and increased spontaneous baroreflex sensitivity; 3) TA correlates with increased resting blood pressure, increased wall-to-lumen ratio of the thoracic aorta, decreased EC50 of phenylephrine-induced contractility in the thoracic aorta, and increased norepinephrine content in the heart.

3.2 METHODS

All animal handling and experimental procedures were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee and conformed to the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011).

3.2.1 Animals

Eight pairs of male and female Wistar-Kyoto rats were purchased from Charles River Laboratories (Kingston, NY). Upon arrival rats were housed 2-3 per cage of the same sex and strain in a temperature-controlled animal housing facility with a 12/12 h light-dark cycle with the lights on at 6:00 a.m. Following a one week acclimatization period, male and female pairs were mated to generate the pups used in the experiment. From postnatal (P) day 1 through P14, we initiated a separation procedure modeled after Plotsky and Meaney (Plotsky & Meaney, 1993) and as previously described (Clinton et al., 2014). Between P1-P14 newborn pups experienced either maternal separation (MS) or neonatal handling (NH). The pups that experienced MS were separated as an entire litter for 180 min. daily between 8:30 a.m. – 12:00 p.m.. The pups that experienced NH were handled in the same manner, except their daily separations were 15 min. in duration. For each dam the entire litter was transferred to different room in a small cage and placed on a heating pad (~37°C), whereas the dam remained in the home cage. All littermates remained in close contact throughout the separation period, and were returned to the home cage after the conclusion of separation. After the final separation on P14, litters remained undisturbed until weaning on P21 at which time male pups were separated and group housed (3 per cage). Rats with the same ELE (i.e. NH or MS) were housed together and were allowed to develop to adulthood undisturbed, except to be weighed each week and for the standard cage changes until P60. Between P60-P70 the rats were tested on a batter of behavioral tests to assess their anxiety-like,

depressive-like, and social behaviors. Results of these experiments have been previously published (Rana et al., 2015).

Four weeks after the conclusion of the behavioral testing, between ~P101-P109, n = 10 MS and n = 10 NH rats were randomly chosen and instrumented with cardiovascular radiotelemetry probes (PA-C40, DSI International).

3.2.2 Radiotelemetry Surgery

Anesthesia was induced with 5% isoflurane and maintained at 2.0-2.5% in 1 L/min O_2. Surgical plane of anesthesia was verified by the absence of limb withdraw to painful pinch stimuli. Using asceptic techniques blood pressure transducers (PA-C40; Data Sciences International, St. Paul, MN) were implanted into the abdominal aorta and glued in place with surgical glue (Vetbond). The body of the telemetry device was sutured into the abdominal wall with silk suture, and the incision was closed with monofilament suture. Prior to the first incision rats were injected with carpofen (5 mg/kg s.c.) and buprenorphine (0.1 mg/kg s.c.) for pain control. Immediately following the surgery, recovered from anesthesia in a warm, clean cage with water provided in a petri-dish. Afterwards, they were then individually housed and recovered for a week before commencement of cardiovascular recordings. During this recovery period animals were checked for signs of distress, such as failure to gain weight, spiked coat, nasal discharge, and decreased water and food intake. They were injected with buprenorphine (0.1 mg/kg s.c.) if necessary, and topical triple antibiotic

ointment was applied to the skin suture wound on a daily basis until complete recovery.

3.2.3 Data Acquisition and Analysis

Blood pressure and activity counts were acquired using Dataquest ART 4.3 software (Data Sciences International). Continuous 24-hour blood pressure recordings were made at specific timepoints during development: P130, P179, P193, P207, P221, and P277. Rats were kept undisturbed without any human interference during these recording sessions. Blood pressure signal was sampled at 500 Hz, while activity signal was based on transmitter signal strength changes that provided an approximate index of overall activity and was collected at 250 Hz.

Mean arterial (MAP), systolic (SBP), and diastolic (DBP) pressures along with heart rate (HR) were extracted from the blood pressure signal. Raw data were sampled at 500 Hz and initially binned into 10-sec averages, which were then used to calculate one-hour moving averages for each parameter. Baseline cardiovascular parameters and activity were analyzed separately for the 12-hr dark/active period and the 12-hr light/inactive period.

3.2.4 Heart rate variability (HRV) analysis (time domain)

HRV was calculated from the continuous blood pressure recordings that were binned into 5-min. continuous segments as recommended by the Task

Force of the European Society of Cardiology the North American Society of Pacing Electrophysiology (1996). A total of 288 segments were collected over 24 hrs. Data were analyzed separately for the dark and light cycles, with 144 segments for each 12 hr. period. HRV was calculated as standard deviation of inter-beat-interval (SDNN) during the each of the 5-min. segments. Data were analyzed separately for the light and the dark phase of the 24-hr cycle.

3.2.5 Spontaneous baroreceptor reflex analysis

DSI data files were imported into the Hemolab Analyzer software (http://www.haraldstauss.com/HemoLab/HemoLab.php) for spontaneous baroreceptor reflex sensitivity (sBRS) quantification. For this analysis we selected two recording segments during the light/inactive phase: between 06:00 a.m. – 12:00 p.m. and between 12:00 p.m. – 6:00 p.m., and two segmentsduring the dark/active phase: between 6:00 p.m. – 12:00 a.m. and between 12:00 a.m. – 06:00 a.m.. Analysis segments that were 30 min in duration were selected during each of these four periods when activity counts were minimal and stable, indicating that animals were quietly resting. Blood pressure data within segment was visually inspected and artifacts were manually removed. Hemolab Analyzer software utilizes the sequence method to calculate sBRS as first described by Bertinieri et.al. (Bertinieri et al., 1985). The software determines baroreflex gain by identifying sequences of four or more heart beats where BP and the pulse interval change in same direction. Baroreflex gain is then determined by averaging the slope of linear regressions of the individual sequences; only

individual sequences with the correlation coefficient (i.e. r^2) ≥ 0.8 were included in this analysis. Aberrant BRS sequences due to noise were visually inspected and manually removed from the analysis.

3.2.6 Resident-Intruder Test

Resident-intruder testing was performed between P263-P266 and was based on the methods in previously published reports (Malkesman et al., 2006 ; Koolhaas et al., 2013). Resident rats were those that experienced either MS or NH during early development, were then implanted with radiotelemetry probes as adults, and were single-housed after the surgery. Intruders were age-matched socially-housed (2-3 per cage) WKY male rats that were placed in the resident's homecage for 10 min. Testing was performed in the early afternoon, when the resident was allowed to rest quietly in his homecage for 1-2 hrs prior to testing. Afterwards, the intruder conspecific was placed into the resident homecage for 10 min. The encounter was recorded and digitized, and was subsequently scored by an experienced observer who was blinded to the treatment groups. Rats were closely observed to ensure that no physical injuries resulted from the encounter; despite aggressive displays by some of the rats none of the animals exhibited physical signs, e.g. bite marks, bleeding, etc. At no time did the rats need to be separated because of potential for serious physical harm (e.g. bites, bleeding). Resident and intruder behavior was quantified in the: social, non-social, and aggressive domains using published methodology (Koolhaas et al., 2013). Several behaviors including social, non-social, offensive, and defensive

behaviors were quantified for the resident (test) rats as well as intruder rats. Social exploration (i.e. exploration of the counterpart without any signs of aggression) and non-social exploration (exploration of the cage) were scored for resident rats as well as intruder rats. Other behaviors that were quantified for resident rats were: clinch attack (i.e. pinning the counterpart down into a submissive posture), ano-genital sniffing (i.e. sniffing genitalia of the counterpart as investigatory behavior), lateral threat (i.e. lateral movement toward the counterpart preceding an attack), rest/inactivity, and grooming behavior. Behaviors that were quantified for intruder rats included: submissive posture (prolonged supine position following attack), move away (moving away from resident rats following threat), rearing (i.e. assumption of an upright posture using only the hind limbs), upright posture (i.e. standing on hind limbs when interacting with the resident rats), and freezing (i.e. prolonged duration of immobility) behaviors.

3.2.7 Tissue and Blood Collection

At the end of the experiment, between the 8:00 a.m. – 12:00 noon rats were anesthetized with 5% isoflurane isofluorane (maintained at 2.0-2.5%) in O_2 delivered at 1.0 L/min. Following thoracotomy, blood was collected from the left ventricle by a puncture with a large gauge needle into EDTA-coated tubes. Whole blood was then centrifuged at 2000x g for 15 minutes to collect plasma, which was stored at −80°C until further analyses. Following the blood collection, rats were rapidly decapitated using a sharp guillotine, and a 1-2 cm segment of

the thoracic aorta was harvested and stored in the Krebs-Henseliet buffer for the vessel-response study that was conducted later that same day. Left ventricle was freshly dissected from the heart and then weighed prior to being flash frozen at -30°C in isopentane. Tissue samples were then stored at -80 °C until further processing.

3.2.8 Vascular reactivity

Isometric tension was measured in isolated thoracic aortic ring segments of WKY rats. Fat and adhering tissue was excised and cleansed from the aortic segments after the tissue harvest. The vessel was cut into individual ring segments (2–3 mm in width) and suspended from a force-displacement transducer in a tissue bath. Ring segments were bathed in Krebs-Henseleit buffer of the following composition (mM): 118 NaCl, 4.6 KCl, 27.2 $NaHCO_3$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1.75 $CaCl_2$, 0.03 Na_2EDTA, and 11.1 glucose. Buffer was maintained at 37°C and aerated with 95% O2-5% CO_2. A passive load of 2 g was applied to all ring segments and maintained at this level throughout the experiment. At the beginning of each experiment, indomethacin-treated ring segments were depolarized with KCl (70 mM) to determine the maximal contractile capacity of the vessel. Rings were then thoroughly washed with Krebs-Henseleit buffer and allowed to equilibrate. In subsequent experiments, the contractile response of vessels was tested by cumulative addition of phenylephrine (PE: $1x10^{-9}$ to $3x10^{-6}$). Real time data were collected for all experiments and downloaded to an IBM PC for analysis. Dose-response profiles

were normalized by calculating the contractile response at each concentration of PE as a percentrage of the response to 70 mM KCl. The effective concentration of PE eliciting 50% of the maximum response (EC50) was calculated and tested for differences between treatment groups.

3.2.9 Vascular structure

Thoracic aortae were sectioned into 10-15 mm segments and fixed in 10% formalin at room temperature for structural studies. Tissue samples were dehydrated through a series of graded ethanols, defatted in xylene, paraffin embedded, and sectioned on the microtome at 20 µm. Sections were stained with hematoxylin and eosin using a standard protocol. Tissue was examined using an Olympus BX61 microscope (Olympus America, Center Valley, PA) outfitted with motorized stage (96S100LE; Ludl Electronic Products, Hawthorne, NY) and a cooled mono CCD camera (Orca R2; Hamamatsu Corporation, Middlesex, NJ). Tissue sections were digitized using CellSens software (Olympus America) under a 10x objective. To calculate the wall-to-lumen ratio, we quantified the total media-intima area and the total area of the aortic lumen within a randomly selected section of the thoracic aorta.

3.2.10 Catecholamine Quantification

Norepinephrine content in the left ventricle and in plasma was quantified using HPLC by the Vanderbilt University's Neurochemistry Core

(https://medschool.vanderbilt.edu/vbi-core-labs/neurochemistry-core; Nashville, TN).

3.2.11 Statistical Analysis

Pearson's chi-squared test was utilized to evaluate the association between TA and ELE. Repeated measure analysis using linear mixed models was performed to examine the associations between ELE, TA and the outcome variables (HR, SDNN, sBRS, MAP, SBP, DBP, and activity) over time, where ELE and TA were treated as fixed factors. An appropriate covariance structure was selected for the model based on initial assessments of the covariance estimates and the goodness-of-fit indices. A likelihood ratio test was used to confirm the best fitted model with the most suitable covariance structure. Pearson's r correlation was used to analyze the correlations between thoracic wall-to-lumen ratio and SBP, and between thoracic wall-to-lumen ratio and DBP. Contractility of the thoracic aorta in response to exogenous phenylephrine application was analyzed using dose-response curves, which were used to extract $\log EC_{50}$ values for further analysis. All other analyses were analyzed with a two-tailed Student's t-test if the data were normally distributed, or with the two-tailed Mann-Whitney test if they were not. Normality of data was tested with the D'Agostino & Pearson omnibus test. Analyses were conducted using SPSS version 22.0 (IBM) and GraphPad Prism 6 (http://www.graphpad.com/). Results are presented as mean ± SEM, and significance level was set at $p < 0.05$.

3.3. RESULTS

3.3.1. Resident-Intruder (RI) test

Upon introduction of the intruder rat into their homecages, resident rats actively explored the new animal. This manifested as active approach, sniff, and, in some cases, attack of the intruder. At the same time the intruder rats explored the novel environment of the new cage by moving around, rearing, sniffing, and to some extent exploring the resident rat. Only some of the resident rats exhibited clinch attack behavior, which is characteristic of the territorial aggressive behavior in rodents (Nelson & Trainor, 2007). Other residents did not exhibit clinch attack, though some manifested lateral threat behavior, and none of the intruder rats exhibited aggressive behaviors. All of the intruder animals that were attacked by residents adopted a submissive posture. One-half of MS (n = 5) and one-half of NH (n=5) animals exhibited clinch attack and were classified as trait aggressive (TA). The remaining animals from the MS (n =5) and NH (n = 5) groups did not show clinch attack and were classified as non-aggressive (NA), indicating that ELE did not impact expression of aggression ($\chi^2 = 0$, $p = 1$).

The latency to clinch attack by TA rats was 151.0 ± 22.0 seconds, with the average frequency of clinch attacks of 2.3 ± 0.4 and a duration of 7.1 ± 1.0 seconds per attack. Intruder rats placed with TA rats exhibited submissive posture frequency of 1.8 ± 0.4 with an average duration of 33.0 ± 9.0 seconds. TA rats also demonstrated decreased levels of non-social exploration ($t_{(18)} = 2.19$, $p = 0.042$) along with increased duration of rest and inactivity ($t_{(17)} = 2.86$, $p = 0.011$; Table 3.1). The intruder rats paired with TA residents spent significantly

more time in submissive posture (U = 4, p < 0.0001), longer duration in move away behavior (U = 17.5, p = 0.036), and less time in non-social exploration ($t_{(18)}$ = 2.69, p = 0.015; Table 3.1).

	Resident Rats			Intruder Rats		
Behavior Duration (sec)	**TA**	**NA**	***p***	**TA**	**NA**	***p***
Social Exploration	135.0±19	139.4±15	0.857	75.9±16	47.6±11	0.153
Non-Social Exploration	**130.2±24**	**211.9±33**	**0.042***	**104.1±25**	**219.8±35**	**0.015***
Anogenital Sniffing	35.21±6	27.74±6	0.340	—	—	—
Lateral Threat	1.5±1	4.6±3	0.970	—	—	—
Rest Inactivity	**89.5±25**	**13.0±5**	**0.011***	—	—	—
Grooming	55.4±20	119.0±30	0.091	—	—	—
Submissive Posture	—	—	—	**183.6±50**	**1.7±1**	**< 0.0001***
Move Away	—	—	—	**5.4±2**	**0.6±0.4**	**0.036***
Rearing	—	—	—	14.0±5	31.8±8	0.063
Upright Posture	—	—	—	16.9±6	6.8±2	0.187
Freezing	—	—	—	156.3±44	187.8±43	0.615

Table 3.1. Behavioral characterization of resident and intruder rats. Resident rats were classified as trait aggressive (TA) if they displayed clinch attack; if not, they were classified as non-aggressive (NA). Behaviors of these two types of residents and of their corresponding intruders were quantified separately. Data are presented as mean ± SEM. Statistically significant differences (p < 0.05) are shown in bold.

3.3.2. Resting Heart Rate

Resting heart rate (HR) was analyzed by treating ELE (MS vs. NH) and aggression (TA vs. NA) as fixed factors and age as a repeated measure using linear mixed model analysis. Data are presented separately to highlight the

effects of ELE and aggression. MS-exposed rats showed lower HR throughout their life both in the light (inactive) and dark (active) phases of the 24 hr cycle. In the light phase, there was significant main effect of ELE ($F_{(1,24.3)} = 6.1$, $p = 0.02$; Fig. 3.1A), with MS rats having and average HR of 279.0±2.5 bpm vs. 287.5±2.5 bpm for the NH group ($p < 0.05$; Table 3.2). Significant main effect of ELE ($F_{(1,17)} = 10.8$, $p = 0.004$; Fig 3.1B) was also observed during the dark phase, with mean values of 310.3 ± 2.1 bpm for the MS group and 319.9 ± 2.1 bpm for NH ($p < 0.01$; Table 3.2). In contrast, no effect of aggression on resting HR was detected either in the light ($F_{(1,24.3)} = 0.4$, $p = 0.55$; Fig 3.1C) or in the dark ($F_{(1,17)} = 1.3$, $p = 0.27$; Fig 3.1D) phase of the 24 hr cycle.

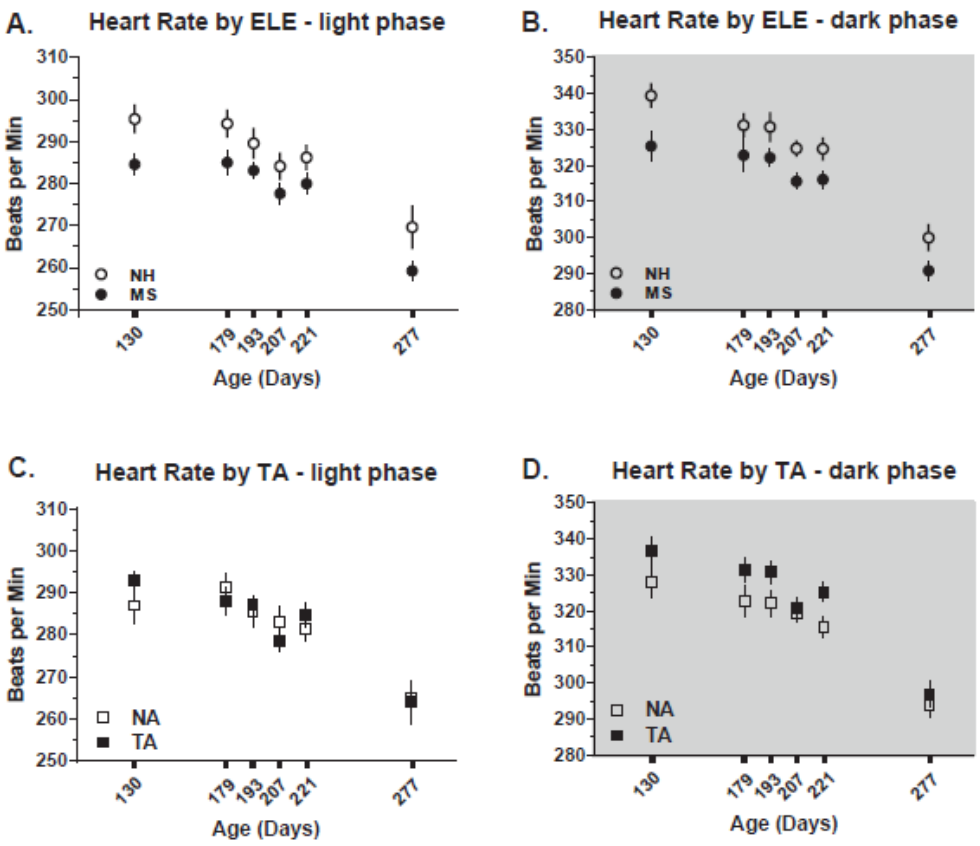

Figure 3.1. Early-life experience impacts on baseline heart rate throughout life but not trait aggression. Wistar-Kyoto rats that were subjected to either neonatal handling (NH) or maternal separation (MS) were implanted with pressure probes for chronic measurement of heart rate in freely moving rats. Radio-telemetry recordings were made throughout the life during following ages (days): 130, 179, 193, 221, and 277. Main effect of early life experience was observed, where MS rats exhibited decreased heart rate as compared to NH rats both in the light (A) and the dark (B) phase throughout lifespan. However, no effect of trait aggression was observed in baseline heart rate both in the light (C) and dark (B) phase throughout lifespan.

3.3.3. Heart Rate Variability and Baroreceptor Reflex Sensitivity

MS-exposed rats showed increased SDNN throughout their life both in the light and in the dark phase. In the light phase, there was significant main effect of ELE ($F_{(1,17.0)} = 5.5$, $p = 0.03$; Fig. 3.2A), but not of aggression ($F_{(1,17.0)} = 0.03$, $p =$

0.87; data not shown) . The mean SDNN was 0.015±0.001 sec for the MS group and 0.013±0.001 sec for NH ($p = 0.03$). Significant main effect of ELE ($F_{(1,17.0)} = 7.5$, $p = 0.014$; Fig. 3.2B), but not of aggression ($F_{(1,17.0)} = 3.9$, $p = 0.06$; data not shown), was also observed during the dark phase. The mean value for SDNN during the dark phase across all ages was: NH=0.011± 0.000 sec vs. MS=0.012±0.000 ($p = 0.014$).

MS-exposed rats also exhibited increased sBRS throughout their life. In the light phase, there was a trend for the main effect of ELE ($F_{(1,17.6)} = 4.3$, $p = 0.05$; Fig. 3.3A), but no significant effect of aggression ($F_{(1,17.5)} = 3.4$, $p = 0.08$; data not shown). The mean value for sBRS during the light phase across the ages was: NH= 2.64±0.18 ms/mmHg vs. MS=3.18±0.18 ms/mmHg ($p = 0.05$). Significant main effect of ELE ($F_{(1,13.3)} = 42.0$, $p < 0.0001$; Fig. 3.3B), but not of aggression ($F_{(1,13.3)} = 0.35$, $p = 0.56$; data not shown), was also detected during the dark phase. The mean value for sBRS during the dark phase across the ages was: NH=2.22 ±0.10 ms/mmHg vs. MS=3.11 ±0.10 ms/mmHg ($p < 0.0001$).

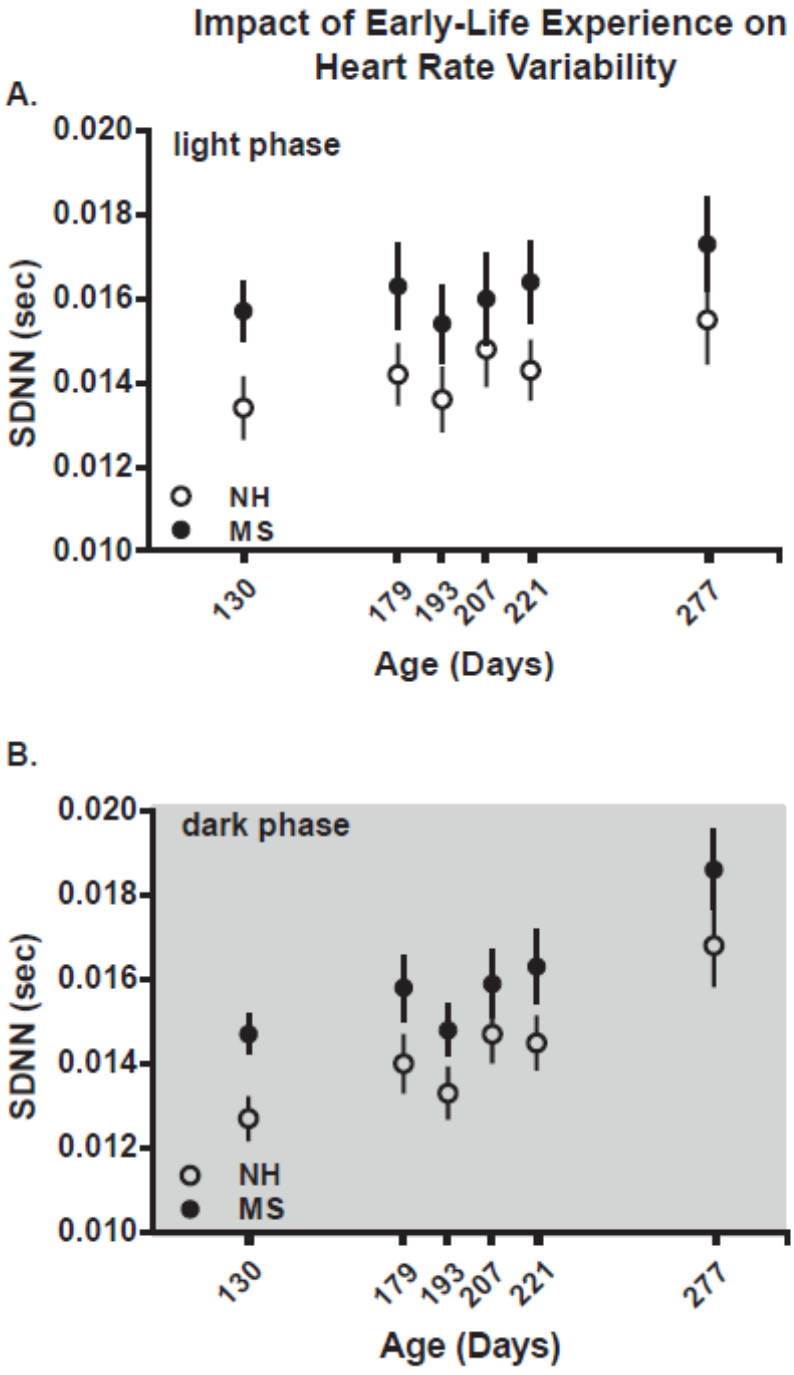

Figure 3.2. Maternal separation increases baseline heart rate variability (HRV) throughout life. HRV was calculated in time domain using standard deviation of inter-beat-interval (SDNN) during light and dark phase throughout the life. Compared to NH rats, MS rats had an increase in SDNN value, during light (A) and dark (B) phase of the 24-hr cycle across the lifespan.

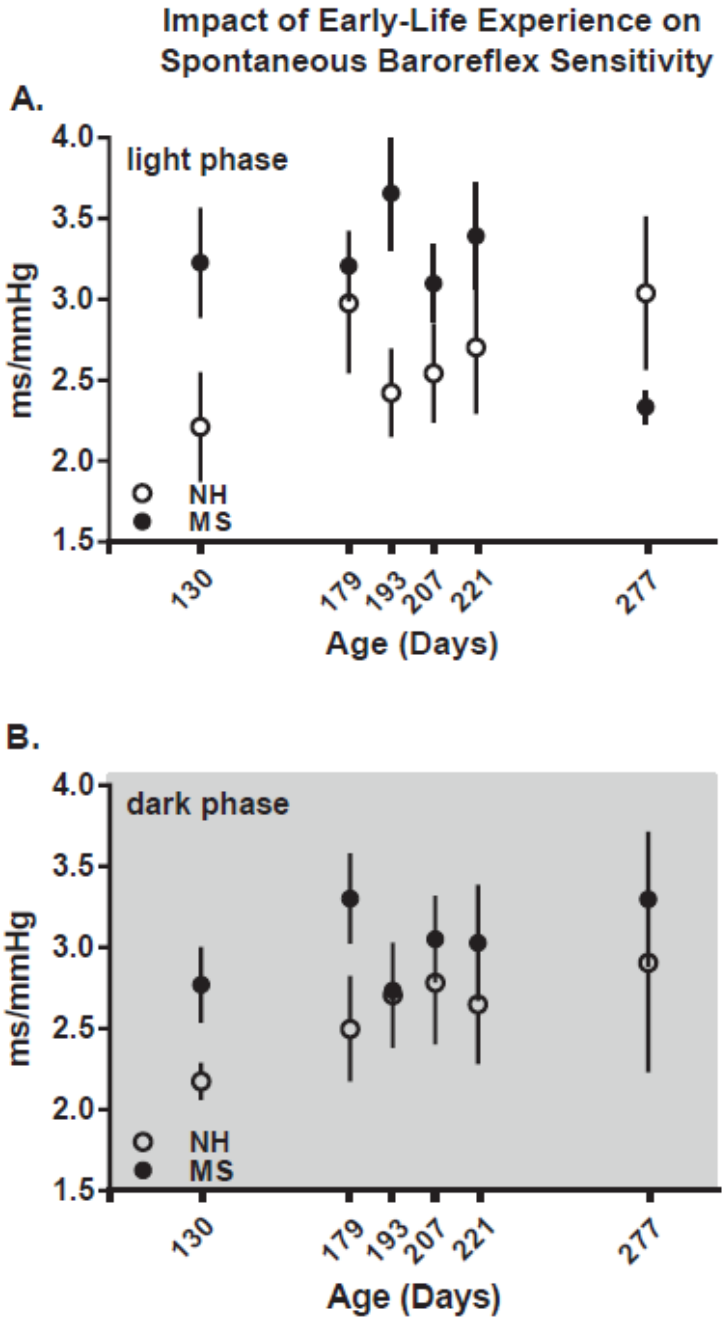

Figure 3.3. Maternal separation increases spontaneous baroreflex sensitivity (sBRS). sBRS was calculated using two 30-min continuous blood pressure data from both light and dark phase at each time-point. There was an overall increase in sBRS gain in MS rats in comparison to NH rats, during light (A) and dark (B) phase across the lifespan.

3.3.4. Baseline Blood Pressure Parameters

MAP was analyzed by treating ELE and aggression as fixed factors and age as a repeated measure using linear mixed model analysis. Data are presented separately to highlight the effects of ELE and aggression. In contrast to resting HR, no effects of ELE on resting MAP were detected during either the light ($F_{(1,17.0)} = 1.8$, $p = 0.19$; Fig. 3.4A) or the dark ($F_{(1,17.0)} = 4.5$, $p = 0.05$; Fig. 3.4B) phase. However, there was a significant effect of aggression during both the light ($F_{(1,17.0)} = 10.8$, $p = 0.004$; Fig. 3.4C) and the dark ($F_{(1,17.0)} = 18.4$, $p < 0.0001$; Fig. 3.4D) phase. During the light phase the mean values for MAP were 109.4 ± 1.5 mmHg for TA and 102.6±1.5 mmHg for NA rats, while during the dark phase these were 107.9 ±1.0 mmHg vs. NA=101.8±1.0 mmHg for TA and NA rats, respectively.

TA rats also exhibited higher SBP throughout their life both in the light and dark phases. There was significant main effect of aggression on SBP in the light ($F_{(1,17.0)} = 6.0$, $p = 0.03$; Fig. 3.5A) and in the dark ($F_{(1,17.0)} = 16.8$, $p = 0.001$; Fig. 5B) phase, with mean values of 132.5 ± 1.8 mmHg (TA) vs. 126.2 ± 1.8 mmHg (NA; $p = 0.03$) during the light phase and 136.1 ± 1.6 mmHg (TA) vs. 126.7±1.6 mmHg (NA; $p = 0.001$) during the dark phase (Table 3.2). In addition, there was a modest, but significant, main effect of ELE on SBP during the dark phase ($F_{(1,17.0)} = 4.8$, $p = 0.04$) of 134.0 ± 1.6 mm Hg vs. 128.8 ± for NH and MS, respectively (Table 3.2). No effect of ELE on SBP during the light phase was detected ($F_{(1,17.0)} = 1.4$, $p = 0.25$).

Similar to SBP, there was a significant main effect of aggression on DBP

both during the light ($F_{(1,17.0)}$ = 6.8, p = 0.019; Fig. 3.5C) and dark phases DBP ($F_{(1,17.0)}$ = 18.3, p = 0.001; Fig. 3.5D) of the 24 hr cycle. Group differences for DBP were approximately 5 mmHg during both the light (90.3 ± 1.3 TA vs. 85.5 ± 1.3 NA; p = 0.019) and the dark (89.4 ± 0.8 TA vs. 84.8 ± 0.8 NA; p = 0.001) phase. There was also a modest effect of ELE on DBP in the dark phase ($F_{(1,17.0)}$ = 4.8, p = 0.04; NH = 88.3 ± 0.8, MS = 85.9 ± 0.8), but not during the light phase ($F_{(1,17.0)}$ = 1.1, p = 0.3).

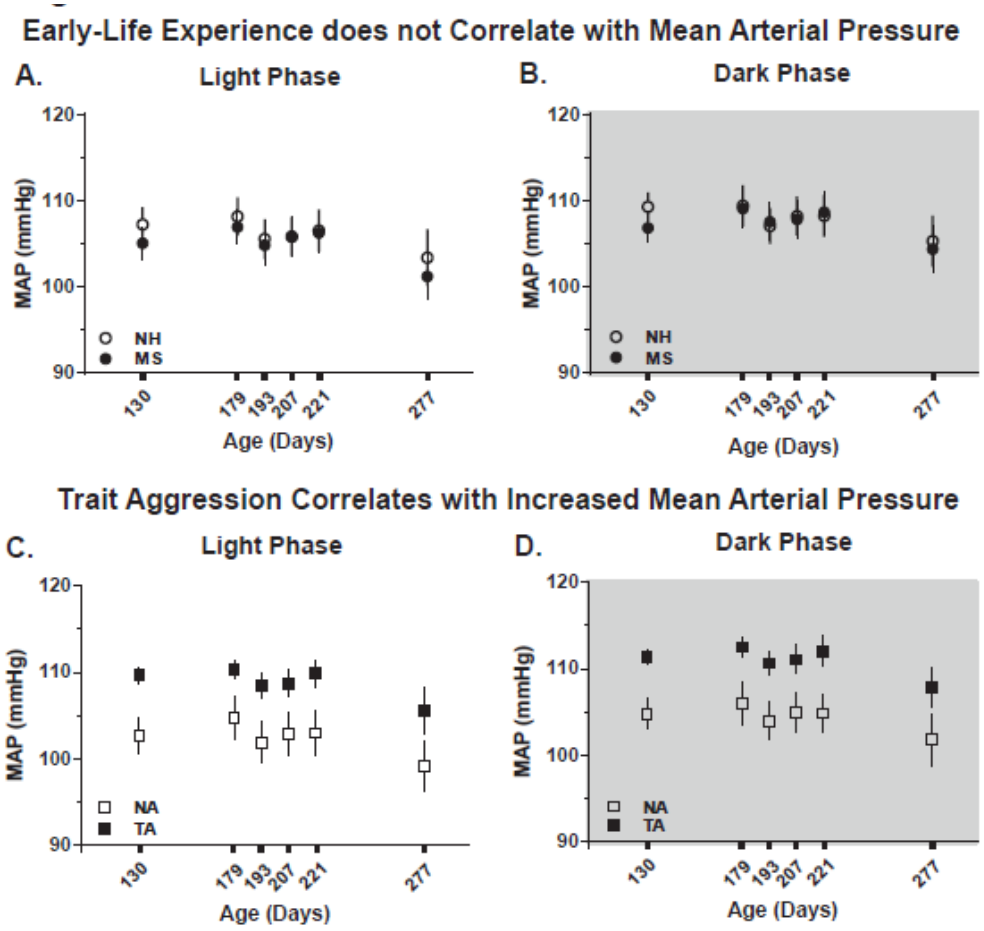

Figure 3.4. Trait aggression impacts on baseline mean arterial blood pressure (MAP) throughout life but not early life experience. Wistar-Kyoto rats that were subjected to either neonatal handling (NH) or maternal separation (MS) were implanted with pressure probes for chronic measurement of heart rate in freely moving rats. Radio-telemetry recordings were made throughout the life during following ages (days): 130, 179, 193, 221, and 277. Aggressive trait was characterized utilizing resident-intruder test at age 263 days. No effect of early life experience was observed in baseline MAP both in the light (A) and dark (B) phase throughout lifespan. However, main effect of trait aggression was observed, where TA rats exhibited increased MAP as compared to NA rats both in the light (C) and the dark (D) phase throughout lifespan.

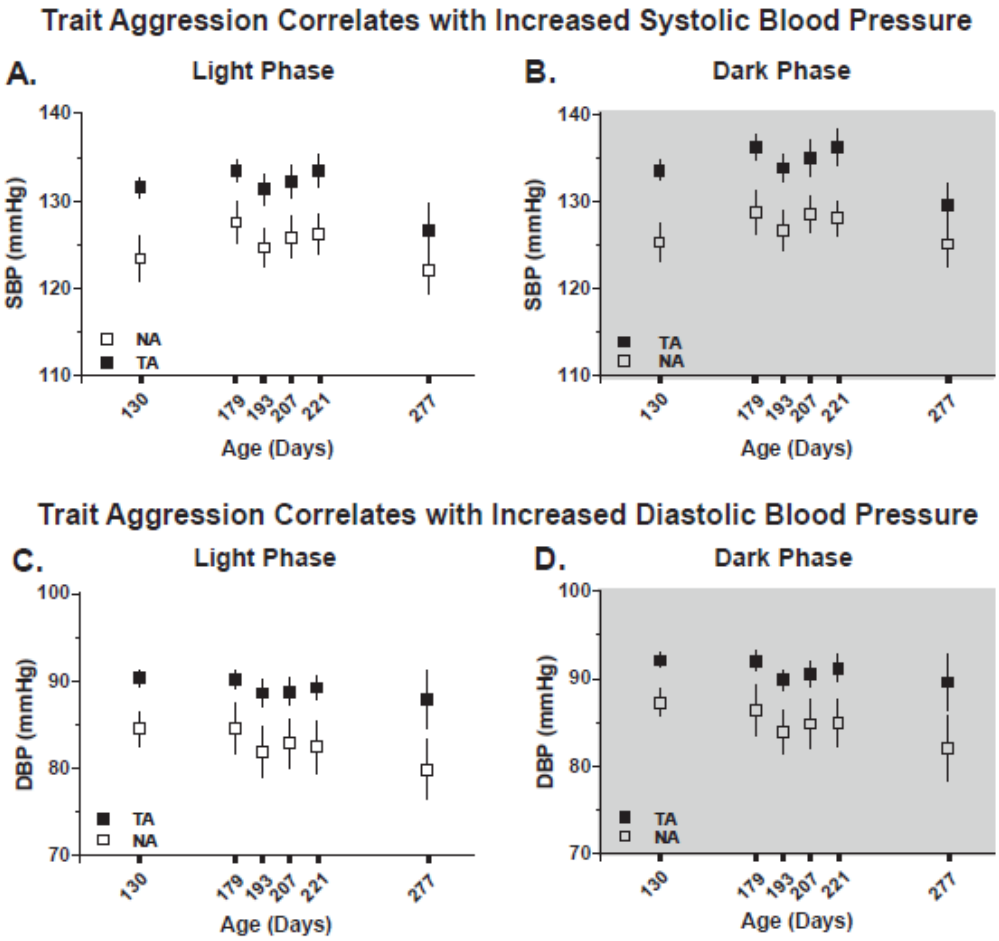

Figure 3.5. Trait aggression correlates with increased baseline systolic (SBP) and diastolic (DBP) blood pressure throughout life. SBP and DBP measures were collected throughout life. Compared to NA rats, TA rats exhibited higher SBP both in the light (A) and dark (B) phase throughout life. Similarly, DBP was also observed to be higher in TA rats as compared to NA rats during light (C) and dark (D) phase across the lifespan.

3.3.5. Baseline Activity

When examining overall activity levels, there was a significant main effect of ELE ($F_{(1,17.0)} = 20.6$, $p < 0.0001$), but not of aggression ($F_{(1,17.0)} = 0.3$, $p = 0.59$), during the light phase with NH rats showing higher activity levels (0.61 ± 0.02 a.u.) in comparison to MS rats (0.50 ± 0.02 a.u.; Table3. 2). In contrast,

during the dark phase there was a significant effect of aggression ($F_{(1,17.0)} = 5.8$, $p = 0.03$), but not of ELE ($F_{(1,17.0)} = 0.1$, $p = 0.76$), with TA rats (2.0 ± 0.05 a.u.) showing increased activity levels in comparison to NA rats (1.8 ± 0.05 a.u.).

	Early Life Experience (ELE)			Trait Aggression (TA)		
Dependent Variable[1] - unit	***NH***	***MS***	***p***	***TA***	***NA***	***p***
HR (light) - bpm	**287.5 ± 2.5**	**279.0 ± 2.5**	**0.02***	284.2 ± 2.5	282.1 ± 2.5	0.55
HR (dark) - bpm	**319.9 ± 2.1**	**310.3 ± 2.1**	**0.004***	316.8 ± 2.1	313.4 ± 2.1	0.27
SDNN (light) - sec	**0.013 ± 0.001**	**0.015 ± 0.001**	**0.03***	0.014 ± 0.001	0.014 ± 0.001	0.87
SDNN (dark) - sec	**0.011 ± 0.000**	**0.012 ± 0.000**	**0.014***	0.011 ± 0.000	0.012 ± 0.000	0.06
sBRS (Light)[2] - ms/mmHg	2.64 ± 0.18	3.18 ± 0.18	0.05	2.67 ± 0.18	3.15 ± 0.18	0.08
sBRS (Dark) - ms/mmHg	**2.22 ± 0.10**	**3.11 ± 0.10**	**<.0001***	2.62 ± 0.10	2.70 ± 0.10	0.56
MAP (light) - mmHg	107.4 ± 1.5	104.6 ± 1.5	0.19	**109.4 ± 1.5**	**102.6 ± 1.5**	**0.004***
MAP (dark) - mmHg	106.4 ± 1	103.4 ± 1.0	0.05	**107.9 ± 1.0**	**101.8 ± 1.0**	**<.0001***
SBP (light) - mmHg	130.9 ± 1.8	127.8 ± 1.8	0.25	**132.5 ± 1.8**	**126.2 ± 1.8**	**0.03***
SBP (dark) - mmHg	**134.0 ± 1.6**	**128.8 ± 1.6**	**0.04***	**136.1 ± 1.6**	**126.7 ± 1.6**	**0.001***
DBP (light) - mmHg	88.9 ± 1.3	86.9 ± 1.3	0.3	**90.3 ± 1.3**	**85.5 ± 1.3**	**0.019***
DBP (dark) - mmHg	**88.3 ± 0.8**	**85.9 ± 0.8**	**0.04***	**89.4 ± 0.8**	**84.8 ± 0.8**	**0.001***
Activity (light) - a.u.	**0.61 ± 0.02**	**0.50 ± 0.02**	**<.0001***	0.56 ± 0.02	0.55 ± 0.02	0.59
Activity (dark) - a.u.	1.9 ± 0.05	1.9 ± 0.05	0.76	**2.0 ± 0.05**	**1.8 ± 0.05**	**0.03***

Table 3.2. Summary of cardiovascular and activity measurements. Data were analyzed using linear mixed modeling to evaluate main effects of early-life experience (NH vs. MS) and aggression (TA vs. NA). Values in bold represent statistically significant ($p < 0.05$) values. Data are presented as mean ± SEM. 1 – modeling based on unstructured covariance structure, 2 – modeling based on compound symmetry covariance structure, * – $p < 0.05$. Abbreviations: a.u. – arbitrary units, MS – maternal separation, NA – non-aggressive, NH – neonatal handling, TA – trait aggressive.

3.3.6. Vasculature Structure

Our radiotelemetry data indicated strong effects of aggression on resting levels of MAP, SBP, and DBP across both light and dark phases of the 24 hr cycle. The higher levels of these parameters in TA animals appeared to be independent of activity, because these differences persisted in the light phase when there were no significant differences in activity (Table3. 2). Based on these observations, we hypothesized that these group differences would correlate with alterations in the vascular structure and function.

To examine potential alterations in vascular structure, we cut paraffin-embedded transverse sections of the thoracic aorta and then stained them with hematoxylin and eosin. Visual inspection of the specimens suggested increased vascular wall thickness in TA rats (Fig. 3.6A). Quantitative analyses revealed a significant increase in the wall-to-lumen ratio in the thoracic aorta of TA rats as compared to their NA counterparts ($t_{(18)} = 2.24$, $p = 0.038$; Fig. 3.6B). suggestive of arterial thickening in TA rats. Furthermore, wall-to-lumen ratio was correlated with both resting DBP ($r^2=0.29$, $p = 0.014$; Fig. 3.6C) and SBP ($r^2=0.34$, $p = 0.0065$; Fig.3.6D) with an apparent segregation of the TA data points toward higher wall-to-lumen ratios and increased DBP and SBP.

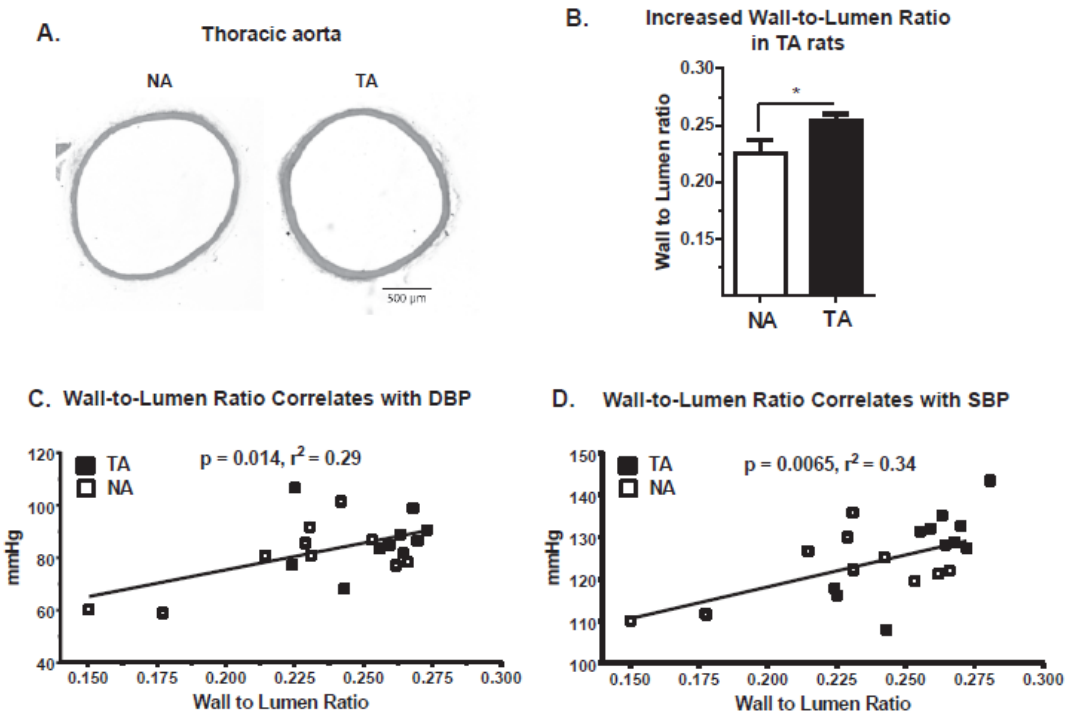

Figure 3.6. Trait aggression correlates with vascular remodeling. Thoracic aortas were extracted, paraffin embedded, and sectioned at a thickness of 20 µm. Sectioned tissues were used for structural measurements using hematoxylin & eosin stain. Representative pictomicrographs of aortas from TA (Ai) and NA (Aii) are presented. Increased wall-to-lumen ratio was observed in TA rats, indicative of arterial thickening (B). When correlated with blood pressure parameters, wall-to-lumen ratio was significantly correlated with diastolic blood pressure: DBP (C), as well as with systolic blood pressure: SBP (D).

3.3.7. Vascular Reactivity

To determine whether increased wall-to-lumen ratio in TA rats was accompanied by differences in vascular reactivity, freshly dissected thoracic aortae were tested for their contractile response to PE, and α1-adrenergic receptor agonist. Examination of contractile responses across a wide range of PE concentrations revealed a leftward shift of the dose-response curve in TA rats (Fig. 3.7A), which corresponded with a significant decrease of the EC50 in TA

rats ($t_{(17)}$ = 3.4, p = 0.0034; Fig. 3.7B).

To determine whether vascular reactivity differences between TA and NA rats may be accompanied by altered levels of norepinephrine in the periphery, we quantified norepinephrine content in plasma and peripheral organs. This analysis revealed a significant increase of norepinephrine content in the left ventricle of TA rats ($t_{(18)}$ = 2.44, p = 0.025; Fig. 3.7C), but no differences in circulating plasma levels ($t_{(18)}$ = 0.68, p = 0.5; Fig. 3.7D).

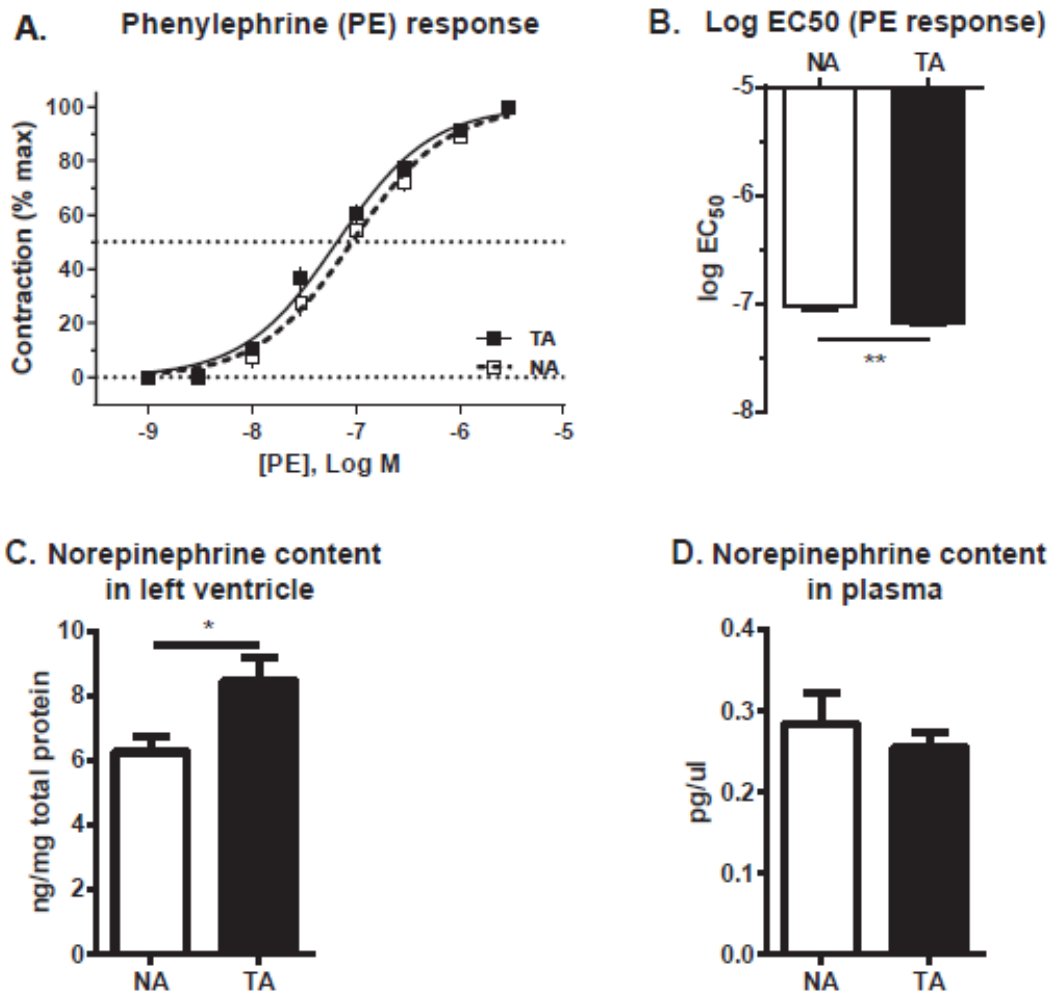

Figure 3.7. Trait aggression correlates with increased response to phenylephrine (PE) along with increased norepinephrine content in left ventricle. Thoracic aortas were freshly dissected and contractile responses to increasing doses of phenylephrine (PE) were measured. Leftward shift of the dose-response curve for aortas extracted from TA rats was observed (A). This corresponded with a significant decrease of logEC_{50} value in TA rats, indicating increased sensitivity of the alpha1-adrenergic receptors (B). Extracted left ventricle and plasma samples were used for the measurement of catecholamine levels. Increased level of norepinephrine was observed in left ventricle of TA rats compared to NA rats (C). However plasma level of norepinephrine was not different between groups (D). * - $p < 0.05$

3.4 DISCUSSION

Data presented in this study suggest that ELE and TA are independent factors that impact cardiovascular system in distinct ways. Specifically, our data indicate that ELE can program resting HR, HRV, and sBRS throughout the lifespan, because MS animals had decreased HR along with increased SDNN and sBRS up to about nine months of age (the longest timepoint in our study). In contrast, ELE did not appear to have a strong effect on blood pressure parameters, with the exception of modest increases in SBP and DBP during the dark phase in NH rats (Table 3.2). However, blood pressure parameters appeared to be strongly influenced by aggression, so that TA rats had significantly elevated MAP, SBP, and DBP throughout the 24 hr cycle. Conversely, aggression did not influence HR, SDNN, or sBRS, because these values were not statistically different between TA or NA rats (Table 3.2). These cardiovascular differences did not appear to be activity-dependent, because significant effects of ELE on HR, SDNN, and sBRS were observed both during the dark and the light phases, while activity differed between NH and MS rats only during the light phase (Table 3. 2). Likewise, TA rats exhibited higher MAP, SBP, and DBP levels throughout the 24-hr cycle, while statistically significant differences in their activity were detected only during the dark period (Table 3.2). We previously reported that MS-exposed WKY rats manifest decreases in depressive- and anxiety- like behaviors as compared to their NH-exposed counterparts (Rana et al., 2015). The present data extend these observations and demonstrate adaptive cardiovascular effects of MS in WKY rats.

In present study we did not detect an influence of ELE on the emergence of TA, because exactly one half of MS-exposed animals were classified as TA and one half of NH-exposed animals were classified as TA (with the remaining animals classified as NA). Previous literature has documented a complex relationship between ELE and the emergence of aggressive behaviors, which is determined by multiple factors that include the exact nature of ELE, rat or mouse strain, and potentially inherent levels of stress-susceptibility. MS in Wistar has been reported to lead to increased offensive play-fighting behavior during the juvenile period along with increased aggression toward a novel conspecific in adulthood (Veenema et al., 2006 ; Veenema & Neumann, 2009). In contrast, MS exposure in the Long-Evans rats does not lead to significant differences in juvenile aggressive behavior (Zimmerberg & Sageser, 2011). Furthermore, work in mice has demonstrated suppression of intermale aggressive behavior in C57BL/6 mice together with increases in anxiety- and depressive- like behaviors as a consequence of MS exposure (Tsuda et al., 2011). Taken together these data indicate a complex interplay between genetic endowment and the environment, which determines whether ELE shapes aggressive behavior later in life. We are not aware of any other studies examining this issue in WKY rats. Our published behavioral data and those from the current study indicate that MS (as compared to NH) diminishes depressive- and anxiety- like behaviors in WKY rats (Rana et al., 2015) and does not impact expression of TA.

Studies in clinical populations have demonstrated the importance of baseline cardiovascular parameters as prognostic factors for future morbidity and

mortality. For example, data from 5,000 participants in the Multi-Ethnic Study of Atherosclerosis (MESA) trial found that for each 1 bpm increase in resting HR, there was a 4% increase in the risk of heart failure and left ventricular dysfunction (Opdahl et al., 2014). Similarly, investigation in 2,501 subjects from the Framingham heart study reported that a reduction in baseline HRV was associated with a significant increase in the risk of cardiac events, including: angina pectoris, myocardial infarction, coronary heart disease death, or congestive heart failure (Tsuji et al., 1996). In addition, a study in 808 patients who survived a myocardial infarction reported that a decrease in SDNN was the strongest predictor of mortality among other factors quantified from Holter monitoring data, including presence and frequency of ventricular premature complexes and the R-R interval (Kleiger et al., 1987). Similarly decreases in sBRS in survivors of myocardial infarction, in heart failure patients, and in hypertension have been implicated in increased morbidity and mortality in a number of clinical trials (La Rovere et al., 1988 ; Farrell et al., 1992 ; La Rovere et al., 1998 ; Ormezzano et al., 2008 ; Mirizzi et al., 2013). Our observations that MS-exposed rats showed decreased resting HR, along with increased SDNN and sBRS indicate that ELE can program adaptive cardiovascular changes throughout adulthood.

In contrast, TA did not seem to impact these autonomic indices suggesting a different and independent mechanism in modulating cardiovascular function. Instead of resting HR and its associated autonomic indices, TA correlated with significant increases in baseline blood pressure parameters, including: MAP,

SBP, and DBP. A large meta-analysis from 61 prospective studies involving one million patients established a strong and direct relationship between resting SBP and DBP and overall mortality rates (Lewington et al., 2002). Conversely, lowering of blood pressure reduces morbidity and mortality due to cardiovascular causes, where a long-term decrease of 5-6 mmHg in baseline DBP was associated with a 35-40% reduction in the incidence of stroke and a 25% reduction in the incidence of coronary heart disease (Collins et al., 1990). These data suggest that even a subtle but persistent difference in blood pressure can have a major impact one cardiovascular health. Thus, the increases in blood pressure parameters associated with TA (i.e. 6-7 mmHg for MAP, SBP and DBP) throughout life suggest poor cardiovascular health, which may confer an increased risk of cardiovascular events in TA rats.

Apart from baseline hemodynamic parameters, structural and functional characteristics of vasculature are also important to stratify the risk and progression of cardiovascular disease. It is known that high blood pressure leads to arterial thickening and is a major risk factor for cardiovascular events such as stroke, myocardial infarction, and other vascular diseases. In addition to increased blood pressure, TA rats showed arterial thickening in thoracic aorta evident by higher wall-to-lumen ratio suggestive of vascular remodeling. Similar results have been observed in clinical settings, where a large community-based study reported that individuals with antagonistic and aggressive traits have greater increases in arterial thickening, which was independent of traditional cardiovascular risk factors (Sutin et al., 2010). Other studies have documented a

significant correlation between trait anger and intima-media thickness in the carotid arteries in both men and women (Bleil et al., 2004 ; Ohira et al., 2012). In one of these studies the authors also reported that depressive symptoms and trait anxiety did not impact arterial thickness (Ohira et al., 2012). This is consistent with our observations that TA is not impacted by ELE (unlike depressive- and anxiety- like behaviors (Rana et al., 2015)), and that this trait correlates with increased wall-to-lumen ratio in rats. Since vascular remodeling of small and large vessels in arterial hypertension may represent early signs of atherosclerosis and small-vessel disease in peripheral organs (Harazny et al., 2007), the observed increase in wall-to-lumen ratio in TA rats suggests their increased risk for vascular dysfunction and disease.

In addition to structural alterations, we also detected increased sensitivity to phenylephrine-induced contractility of the thoracic aorta as evidenced by the leftward shift of the dose-response curve and a decrease of the EC_{50} in TA rats. These changes accompanied increases in resting MAP, SBP, and DBP in the TA rats, and are consistent with a previous report of increased vascular sensitivity to PE in the model of ouabin-induced hypertension (Kimura et al., 2000). The decrease in the EC_{50} suggests increased sensitization of α1-adrenergic receptors to PE rather increased receptor density, because the maximal response to PE did not differ between groups. This difference in sensitivity to PE may also be accompanied by other vascular alterations, such as impairment in the endothelium-dependent vasodilation as is common in hypertension (Rossoni et al., 2002). Though we did not evaluate vascular contractility in endothelium-

denuded speciments, it is unlikely that there is TA rats manifest alterations in endothelial function (at least in thoracic aortae), because we did not detect difference in acetylcholine-induced relaxation when compared to NA rats (data not shown).

These differences in receptor sensitivity may also be accompanied by an increase in the sympathetic tone to specific peripheral organs. Due to technical limitations we were not able to quantify norepinephrine content within the aorta or the arterioles. However, we detected an increase in norepinephrine levels in the hearts of TA rats, but not in plasma. While it is often thought that sympathetic drive to different organs is uniform, extensive evidence indicates that sympathetic nerve activity can be engaged in distinct patterns depending on the stimulus or environmental challenge (Kerman et al., 2000 ; Morrison, 2001). Our findings of increased norepinephrine content in the heart, but not plasma, are consistent with this notion and suggest an increase in the sympathetic drive to the heart in TA rats, which may lead to increased cardiac contractility.

In conclusion, the present study highlights the importance of ELE as well as TA in modulating cardiovascular parameters. ELE was observed to impact HR parameters, where MS-exposed WKY rats showed improved baseline function. Our behavioral data indicate that MS has protective effects in the stress-susceptible WKY rats, but leads to adverse changes in the genetically related and stress resilient Wistar rats (Rana et al., 2015). The protective effects of early-life stress may be due to the adaptive responses triggered by MS, which programs the neonates to better adapt to stressful situations in adulthood. Such

a predictive adaptive response is likely mediated by epigenetic mechanisms and has been proposed to be especially prominent in stress susceptible individuals (Gluckman et al., 2007 ; Schmidt, 2011 ; van der Doelen et al., 2013). Future studies aimed at epigenetic effects in WKY rats with differences in ELE will be required to address this notion. On the other hand, TA seems to be an inborn trait that is not impacted by ELE in WKY rats, and which correlates with specific vascular and blood pressure alterations. It is feasible that these effects are mediated by the differential sympathetic activation of the nerves innervating the heart and vasculature. Future work will be required to evaluate this notion.

CHAPTER 4: EFFECT OF METHYL-DONOR SUPPLEMENTATION ON BEHAVIOR AND CARDIOVASCULAR PARAMETERS IN WISTAR KYOTO (WKY) RATS

4.1. INTRODUCTION

Growing evidence highlights the importance of epigenetic mechanisms in mediating gene-environment interaction throughout the lifespan, where epigenome is sensitive to environmental stimuli (Roth & Sweatt, 2011). Histone modifications, DNA methylation, and microRNA induced epigenetic changes are some of the known mechanisms involved in epigenetic regulation of gene expression (McGowan et al., 2008). DNA methylation is one of the epigenetic mechanisms that have been widely studied, where alterations in DNA methylation have been implicated in several diseases, including neuropsychiatric and cardiovascular disorders. Most commonly DNA methylation involves the addition of methyl groups onto the 5' position of the cytosine ring of the CpG dinucleotide sequence (Wainfan & Poirier, 1992). This process is catalyzed by the enzymatic activity of DNA methyltransferases (DNMTs) that transfer the methyl group from the methyl donor S-adenosylmethionine (SAM) (Kim et al., 1995). Repressor proteins such as methyl-binding domain protein MeCP2 and histone deacetylases bind to the methylated cytosines usually suppressing gene transcription (Bird, 2002 ; Miranda & Jones, 2007). However, other studies have

implicated this process in promoting gene expression (Chahrour et al., 2008 ; Cohen et al., 2008).

Epigenetic changes are influenced by a variety of environmental factors such as maternal care, diet, stress, infectious agents, and immunological factors (Duthie et al., 2000 ; McGowan et al., 2008 ; Seery et al., 2010) . Among these diet can play a key role with dietary methyl donors that include folic acid, vitamin B12, choline, betaine, methionine, and zinc (Seery et al., 2010 ; Loria et al., 2015 ; Maniam et al., 2015). SAM (which actively donates methyl groups for DNA methylation) synthesis is dependent on the availability of dietary methyl donors, where methionine is the key precursor molecule (Cohen et al., 2008 ; Chen & Miller, 2012). Increasing evidence suggest a key role for DNA methylation in the etiology of various disease states, including various cancers, neuropsychiatric disorders, and cardiovascular disorders (McGowan et al., 2008 ; Luecken et al., 2009 ; Natarajan & Caramaschi, 2010 ; Roth & Sweatt, 2011 ; Loria et al., 2014).

Previously, we found that exposure to maternal separation in the stress-susceptible Wistar-Kyoto (WKY) rats during the early postnatal period can program behavioral and cardiovascular function throughout the lifespan. These changes were accompanied by an increase in global DNA methylation in the hippocampus but not in other brain regions (Fig. 4.1). The aim of the current study was to determine whether diet-induced increase in DNA methylation can produce adaptive behavioral and cardiovascular changes akin to those induced by maternal separation.

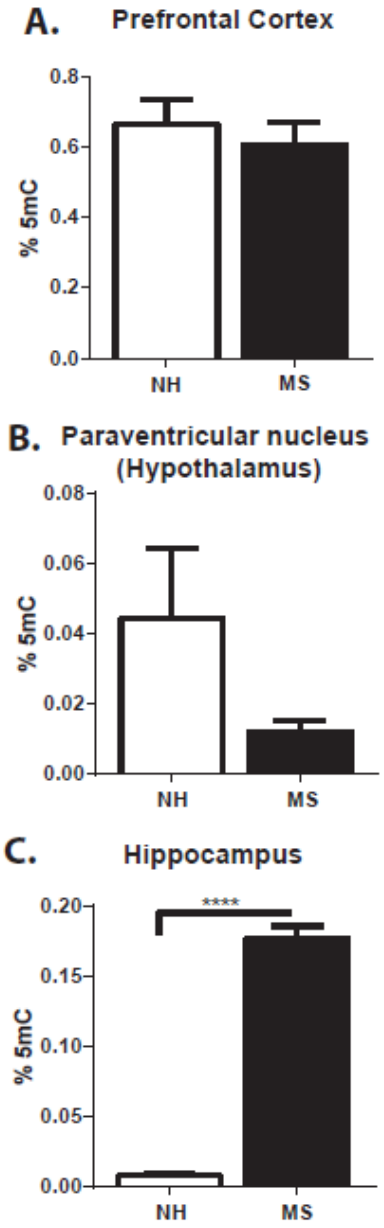

Figure 4.1. Whole genome DNA methylation in various brain regions. DNA from various brain regions of the rats that were previously exposed to either maternal separation (MS) or neonatal handling (NH) were extracted and whole genome methylation was quantified. No difference in DNA methylation was observed in the prefrontal cortex (A; $p = 0.56$) and paraventricular nucleus of the hypothalamus (B; $p = 0.11$) between the two groups. However, selective effect of maternal separation was observed in DNA methylation where increased methylation was observed in the hippocampus of MS rats compared to hippocampal DNA methylation of NH rats (C; $p < 0.0001$ - ****).

4.2. METHODS

All animal handling and experimental procedures were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee and conformed to the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011). Experimental timeline for the experiment is presented in Figure 4.2.

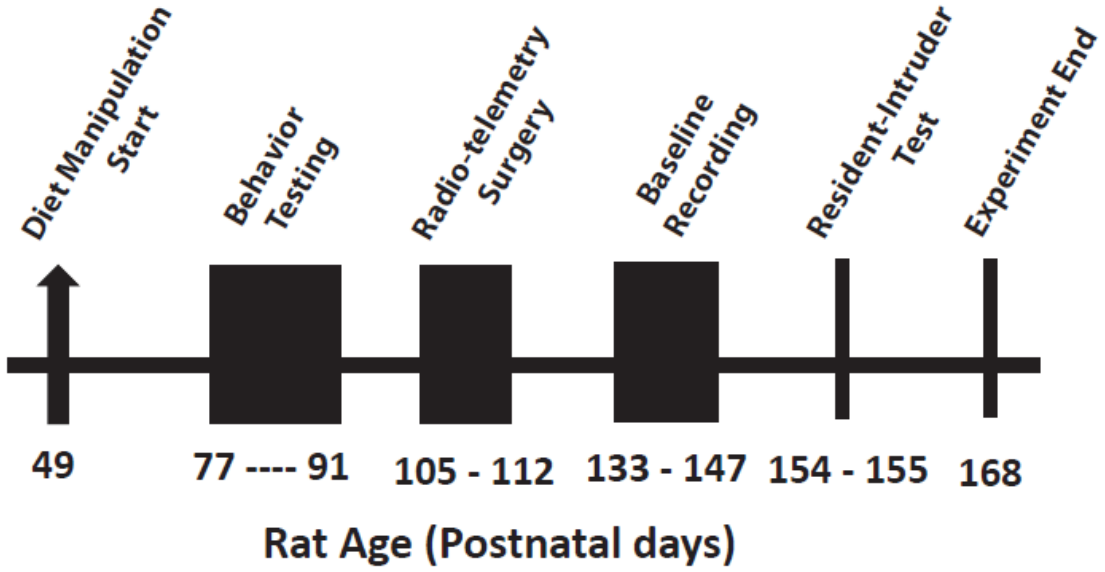

Figure 4.2. Experimental timeline of methyl-donor manipulated diet paradigm. The diagram depicts the overall experimental timeline where numbers on X-axis denotes the rats' (P)ostanatal day age. The solid blocks refer to specific manipulation listed right above it. Diet manipulation was started when the rats were of age P49 and lasted through the entire experiment. After four weeks of diet manipulation, rats were tested on several behavioral tests from P77 – P91. Radio-telemetry surgery was performed to implant pressure probes (DSI International) from P105 - P112. Baseline cardiac parameters were recorded from P133 – P147 after full recovery from the surgery. Resident-Intruder test was performed at the age of P154 – P155. At the end of the experiment, rats were sacrificed and organs were collected for further investigations. *Thicknesses of blocks are not scaled.*

4.2.1 Animals

A total of 28 male Wistar-Kyoto (WKY) rats were purchased from Charles River Laboratories (Kingston, NY, Willmington, MA). Upon arrival rats were housed 2 per cage of the in a temperature-controlled animal housing facility with a 12/12 h light-dark cycle with the lights on at 6:00 a.m. Following a one-week acclimatization period, beginning on postnatal day (P)49 standard rat chow was substituted for either a methyl donor-depleted (WKY-Dep; n = 14) or a methyl donor-supplemented (WKY-Sup; n = 14) diet (Table 1; Research Diets Inc., New Brunswick, NJ); the animals in each experimental group remained on these respective diets for the duration of the study. These diets were either supplemented or depleted with: methionine, choline, folic acid, betaine, vitamin B12, and zinc, all nutrients that are essential for DNA methylation. Behavior testing was conducted from P77 – P91 to assess anxiety-like, depressive-like, and social behaviors. Between P105 – P112 the rats were instrumented with radiotelemetry probes (PA-C40, DSI International) for monitoring of blood pressure and heart rate. At P154 rats were tested on the resident-intruder test.

	Methyl-donor Depleted	Methyl-donor Supplemented
Nutrient	**Content**	**Content**
Methonine (g)	0.6	7.5
Choline (g)	0.0822	15
Folic Acid (mg)	0.2	15
Betaine (g)	0	15
Vitamin B12 (mg)	0.015	1.5
Zinc (mg)	29.05	150

Table 4.1. List of nutrients (methyl-donor) content on methyl-donor depleted vs. methyl-donor supplemented diet. Comprehensive list of all the nutrients can be found on company's website (Research Diets Inc., New Jersey)

4.2.2 Behavior Test Battery

All behavior tests were conducted under dim light conditions (30 lux) between 8:30 a.m. – 11:30 a.m.. Animals were habituated to the testing room by placing home cages in the test room overnight prior to testing; this habituation process was used for all tests except for the forced swim test (FST). Tests were conducted in the following order: open field test (OFT), FST, and social interaction.

4.2.2.1 Open Field Test (OFT)

Testing was conducted in a 100 × 100 × 50 cm black Plexiglas box with a black floor. At the beginning of the test, a rat was placed in a corner of the box and was allowed to explore the apparatus for 5 min. The apparatus was thoroughly cleaned between each test animal. Behavior was recorded with a digital camera, and parameters including total distance travelled, latency to enter

the center of the open field, amount of time spent in the center and periphery were quantified utilizing Ethovision® XT 8.0 video tracking software (Noldus, Wageningen, The Netherlands). A trained observer that was blinded to experimental groups manually assessed grooming and rearing behavior using Ethovision software.

4.2.2.2 Forced Swim test (FST)

This test was adapted from Cryan et al. (Cryan et al., 2005a) and was conducted as previously described (Nam et al., 2014b) . The water chambers were clear Plexiglas cylinders (40 cm high × 40 cm diameter) filled with water at 25 °C to a depth of 30 cm. On FST day 1, rats were placed (one rat/cylinder) in the water for 15 min (pretest phase); 24 h later the rats were returned to the water-filled cylinder and tested for 5 min (test phase). Water was changed after every swim session, and each cylinder was cleaned. Each rat's behavior was digitally recorded, and immobility time was scored by the Ethovision® XT 8.0. We focused on the immobility measure since it is classically considered an indicator of behavioral despair and depressive-like behavior (Porsolt et al., 1977), and it can be clearly defined and easily distinguishable from active coping measures such as swimming and climbing, which are sometimes difficult to reliably distinguish across experimental observers (Porsolt et al., 1977 ; Cryan et al., 2005b).

4.2.2.3 Social Interaction

Social interaction testing was conducted in a rectangular black Plexiglas box (91 × 61×30 cm) with a black floor. The apparatus was divided into three chambers (zones) separated by two black Plexiglas dividers with openings in the center of the dividers to allow test animals to move freely from one zone to another. Testing was conducted over a 10 min period. The test rat was placed in the neutral zone (middle chamber), while one of the other zones contained an empty cylindrical interaction cage with metal bars placed in a corner of the zone. The third zone contained a male stimulus rat within an interaction cage placed in a corner. An approximately 2-cm wide zone around each interaction cage was designated as the interaction zone. The metal bars of the interaction cage allowed rats to interact and sniff each other, but prevented any aggressive encounters between animals. Stimulus rats were age-matched with the test animals. They were habituated to the interaction cages by exposures of 5, 10, and 15 min. on consecutive days. Behavior was recorded with a digital camera and quantified with the Ethovision® XT 8.0 system. Frequency of visits and time spent in the neutral, object, and male zones were assessed.

4.2.3 Radiotelemetry Surgery

At P105 – P112 animals were randomly chosen from the WKY-Sup (n = 7) and WKY-Dep (n = 7) groups for radiotelemetry probe implantation for cardiovascular monitoring. Anesthesia was induced with 5% isoflurane and maintained with 2.0-2.5% isoflurane in oxygen delivered at 1 L/min. Pressure

transducer at the tip of the catheter was implanted into the abdominal aorta and glued in place with surgical glue (Vetbond Tissue Adhesive; 3M, http://solutions.3m.com/), while the body of the device (PA-C40, Data Sciences International, St. Paul, MN) was sutured into the abdominal wall. Aseptic techniques were used throughout all the surgeries, and the animals were injected with carpofen (5 mg/kg; s.c.) and buprenorphine (0.1 mg/kg; s.c.) for pain control prior to surgery. Rats recovered from anesthesia in a warm, clean cage with water provided in a petri dish. They were then single-housed after the surgery and recovered for one week before the first baseline recordings were collected. Daily routine checks for weight, signs of distress (e.g. spiked coat, lethargy, rings around eyes), food and water intake, and excretory functions were performed throughout the recovery period. Animals showing signs of distress were administered buprenorphine (0.1 mg/kg s.c.). Rats were also treated with a topical antibiotic cream applied to the incision site on a daily basis during recovery.

4.2.4 Data Acquisition and Analysis

Following the recovery, rats were housed in recording room equipped with DSI hardware and software (ART 4.3; Data Sciences International). Baseline systolic blood pressure (SBP), mean arterial pressure (MAP), diastolic blood pressure (DBP), heart rate (HR), and locomotor activity were recorded between the ages of P133-P147. Continuous blood pressure recordings were acquired

with a sampling rate of 500 Hz with data averaged over 10 seconds. Rats were kept undisturbed during recording sessions.

Data were extracted from the DSI ART 4.3 analysis software as 30-minute moving average. HR, BP and activity recordings were analyzed separately during the 12 hr light (inactive) and the 12 hr dark (active) phases of the 24 hr cycle.

4.2.5 Heart rate variability (HRV)

Heart rate variability (HRV) was analyzed in the time domain and was calculated from the 24 hr continuous blood pressure recordings that were binned into 5-minute segments. The segment length was selected as per the recommendation from the Task Force of the European Society of Cardiology the North American Society of Pacing Electrophysiology (1996). HRV was calculated using standard deviation of inter-beat-interval (SDNN) during the 5-minute segments. Data was averaged during the 12-hr light phase and 12-hr dark phase and further analyzed.

4.2.6 Response to Intruder Stress

Cardiovascular recordings to intruder stress were obtained at the age of P154 – P155 during the midpoint of the light phase of the 24 hr cycle. Test rats were exposed to age-matched socially-housed intruders for 10 minutes in their homecage. Blood pressure and HR were sampled at 500 Hz and digitized as 10

second moving averages. Data were then averaged over one-minute bins and extracted for offline analyses. Data were acquired for 1-2 hrs before intruder exposure when test rats remained undisturbed to obtain a stable baseline, and then 10 min during and 2 hrs after intruder exposure. Maximal responses for HR and MAP in terms of percent change from baseline were calculated for each animal. In addition, we quantified cardiovascular recovery from maximal responses using curve-fitting analysis. One phase decay for MAP and HR was used as the model to fit the data, using the formula: Y= (Y0 - Plateau)*exp(-K*X) + Plateau, where K is the rate constant/decay constant.

4.2.7 Tissue Collection and Preparation

Rats were rapidly decapitated with a guillotine. The brain and peripheral organs were then extracted, flash frozen and store at -80°C for future analyses. Alternating brain sections cut at 300 µm and 20 µm were then collected. The thin (20 µm) sections were stained with cresyl violet using a standard protocol to guide identification of anatomical landmarks for tissue punches in the adjacent thick (300 µm) sections. Dorsal hippocampus samples were collected from the thick sections using 0.5 mm diameter tissue punch (Harris Micro-Punch, Ted Pella, Redding, CA). A portion of the tissue samples were used for DNA using Qiagen DNeasy Blood & Tissue kit (Qiagen; www.qiagen.com), which included RNase A treatment. DNA samples were subsequently stored at -20°C. Another portion of the hippocampal samples were shipped to the Vanderbilt University

Neurochemistry Core (https://medschool.vanderbilt.edu/vbi-core-labs/neurochemistry-core; Nashville, TN) for amino acid content quantification. Global DNA methylation was assayed using colorimetric Methyl Flash Methylated DNA Quantification kit (Epigentek; www.epigentek.com/) in 100 ng DNA samples per well analyzed in triplicate and compared to a five-point standard curve of methylated DNA (0-10 ng). The fraction of global DNA methylation was calculated using optical density measurements per manufacturer's instruction.

4.2.8 Statistical Analysis

Data were analyzed using GraphPad Prism 6 (http://www.graphpad.com/); normality of data was tested with the D'Agostino & Pearson omnibus test. Data were analyzed with a two-tailed Student's t-test, or with the Mann-Whitney U test if not normally distributed. Blood pressure and HR data averaged over 30 minute intervals were analyzed using repeated measures two-way ANOVA, with time and diet-manipulation treatment as factors. Significance was set at $p < 0.05$, and results are presented as mean ± SEM.

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4.3. RESULTS

A total of 28 WKY rats were used in the present study (WKY-Sup; n= 14 and WKY-Dep; n= 14) for the behavior analysis. A total of 14 rats were used for radio-telemetry study (WKY-Sup; n=7 and WKY-Dep; n= 7). And a total number of 16 rats (WKY-Sup; n= 8 and WKY-Dep; n= 8) were used for hippocampal global DNA methylation and methionine content analysis.

4.3.1. Behavioral effects

The behavioral consequences of the diet manipulation were evaluated on a series of tests to ascertain potential changes in depressive-like, anxiety-like, and social behaviors. At the time beginning of testing the animals had been fed different diets for 4 weeks prior. Compared to their WKY-Dep counterparts, WKY-Sup rats exhibited increased exploration in the novel environment as evidenced by their increases in the overall locomotion ($t_{(26)} = 1.89$, $p = 0.035$; Fig. 4.3A), rearing frequency ($t_{(26)} = 1.77$, $p = 0.045$; Fig. 4.3B), and rearing duration ($t_{(26)} = 1.93$, $p = 0.032$; Fig. 4.3C) in the OFT. On social interaction testing no group differences were observed in the frequency of visits to a novel object ($U = 93.5$, $p = 0.846$; Fig. 4.3D), however, the frequency to visit a novel male was greater in WKY-Sup rats ($t_{(25)} = 2.33$, $p = 0.014$; Fig. 4.3E). On the FST, WKY-Sup rats exhibited decreased duration of immobility ($t_{(25)} = 3.65$, $p = 0.0006$; Fig. 4.3F) and an overall increase in the velocity of movement ($t_{(25)} = 4.76$, $p < 0.0001$; data not shown) as compared to the WKY-Dep rats.

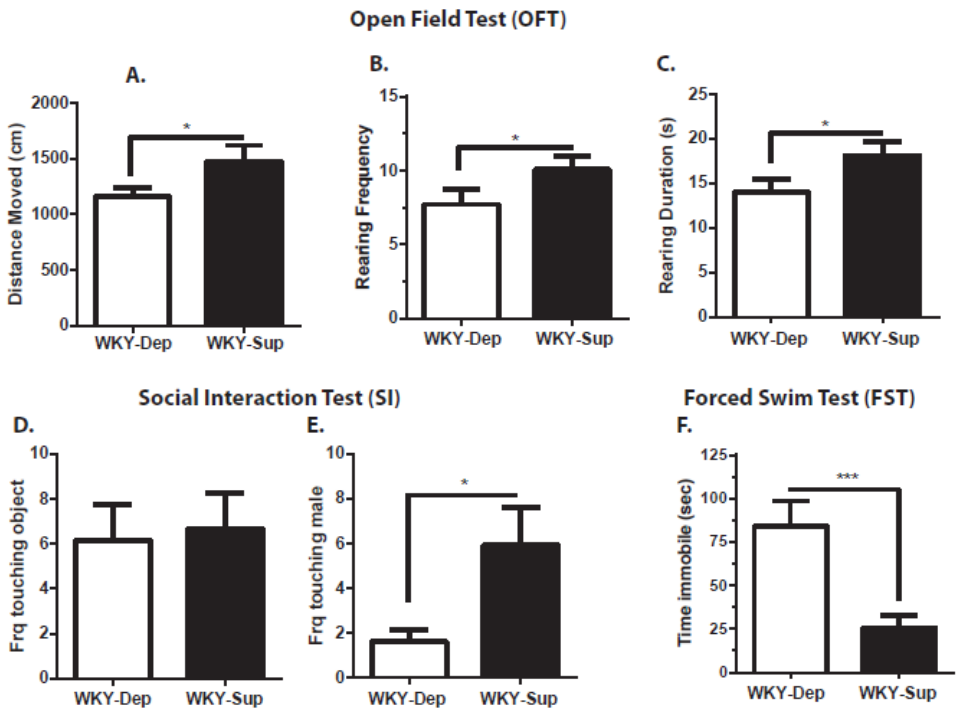

Figure 4.3. Methyl-supplemented diet improves emotional behavior and sociability. WKY rats were tested through behavioral battery after 4 weeks on either methyl-supplemented or methyl-depleted diet. On OFT, WKY-Sup rats displayed increased novelty-induced activity (A), increased rearing frequency (B), and increased rearing duration (C) compared to WKY-Dep rats, indicating decreased novelty induced anxiety-like behavior. WKY-Sup rats also exhibited decreased immobility duration on day 2 of FST in comparison to WKY-Dep rats suggestive of decreased depressive-like behavior (D). Furthermore, when tested on social interaction test, no difference was observed in visit frequency to close proximity to a novel object between groups (E). However, WKY-Sup rats frequently visited in close proximity to a novel male rat compared to WKY-Dep rats, indicative of increased sociability (F). * - $p < 0.05$, *** - $p < 0.001$.

4.3.2. Baseline Cardiovascular Indices

For resting HR, there were significant main effects of time ($F_{(23, 276)} = 7.95$, $p < 0.0001$) and of diet ($F_{(1, 12)} = 80.33$, $p < 0.0001$) during the light phase (Fig. 4.4A). Similarly, there were significant main effects of time ($F_{(23, 276)} = 5.24$, $p < 0.0001$) and of diet ($F_{(1, 12)} = 74.46$, $p < 0.0001$) for resting HR during the dark

phase (Fig. 4.4B). These observations were confirmed when averages across the 12 hr light and dark phases were analyzed, with WKY-Sup rats displaying significantly decreased HR during the light ($t_{(12)} = 8.96$, $p < 0.0001$; Fig. 4.4C) and the dark ($t_{(12)} = 8.63$, $p < 0.0001$; Fig. 4.4D). These changes in resting HR were accompanied by an increase in SDNN during the light ($t_{(12)} = 2.63$, $p < 0.02$; Fig. 4.4E), but the dark ($U = 22$, $p = 0.32$; Fig. 4.4F), phase in WKY-Sup rats.

For SBP, there were significant main effects of time ($F_{(23, 276)} = 2.33$, $p = 0.0008$) and of diet ($F_{(1, 12)} = 15.55$, $p = 0.002$) with WKY-Dep showing higher values (Fig. 4.5A). During the dark phase, there were significant main effects of time ($F_{(23, 276)} = 2.96$, $p < 0.0001$) and of diet ($F_{(1, 12)} = 24.14$, $p = 0.0004$) for SBP with WKY-Dep rats again having higher values (Fig. 4.5B). When the 12 hr averages were analyzed, WKY-Sup rats displayed significantly decreased SBP during the light ($t_{(12)} = 3.94$, $p = 0.002$; Fig. 4.5C) and the dark ($t_{(12)} = 4.91$, $p = 0.0004$; Fig. 4.5E) phases compared to the WKY-Dep rats.

Similar to SBP, there were significant main effects of time ($F_{(23, 276)} = 2.32$, $p = 0.0008$) and of diet ($F_{(1, 12)} = 5.16$, $p = 0.04$) for DBP during the light phase with WKY-Sup rats manifesting decreased values (Fig. 4.5A). During the dark phase, there were also significant main effects of time ($F_{(23, 276)} = 2.69$, $p < 0.0001$) and of diet ($F_{(1, 12)} = 13.77$, $p = 0.003$) with WKY-Sup rats showing lower DBP (Fig. 4.5B). Analyses of the 12 hr averages confirmed these observations and showed significantly decreased DBP during the light ($t_{(12)} = 2.27$, $p = 0.042$; Fig. 4.5D) and the dark ($t_{(12)} = 3.71$, $p = 0.003$; Fig. 4.5F) in the WKY-Sup group.

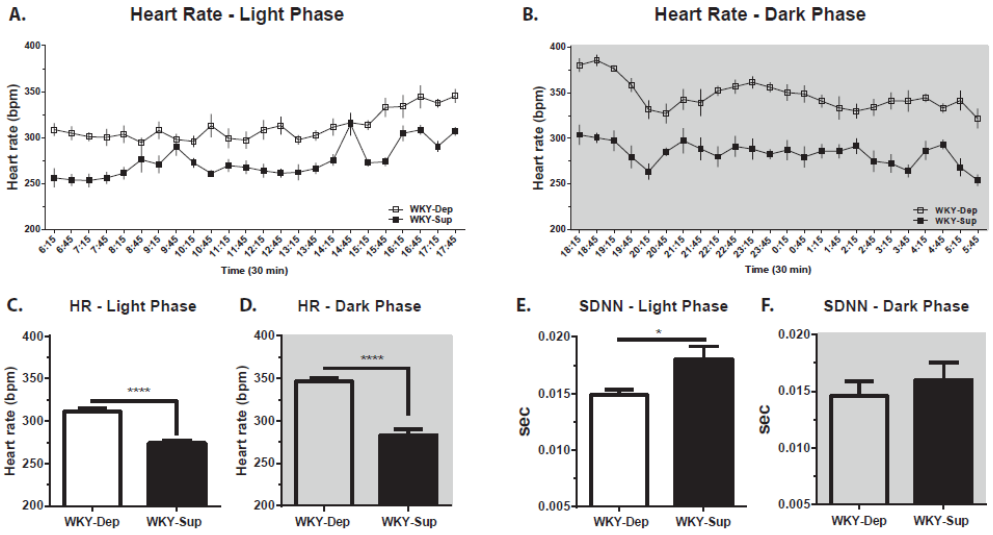

Figure 4.4. Methyl-supplementation lowers resting heart rate (HR) during light and dark phase and increases diurnal resting heart rate variability (HRV). HR values were either averaged for 30-min interval throughout light/resting and dark/active phase (A, B) or over the entire light/resting and dark/active phase (C, D). WKY-Sup rats displayed lower resting HR throughout the light phase (A), which was also significantly lower when overall average of the light phase was compared to WKY-Dep rats (C). Similarly, WKY-Sup rats exhibited lower resting HR throughout the dark phase (B), which was also significantly lower when overall average of the dark phase was compared to WKY-Dep rats (D). HRV was calculated in time domain using standard deviation of inter-beat-interval (SDNN) during light and dark phase. Compared to WKY-Dep rats, WKY-Sup rats had an increase in SDNN value, during light (E) but not during the dark (F) phase of the 24-hr cycle. * - $p < 0.05$, **** - $p < 0.0001$.

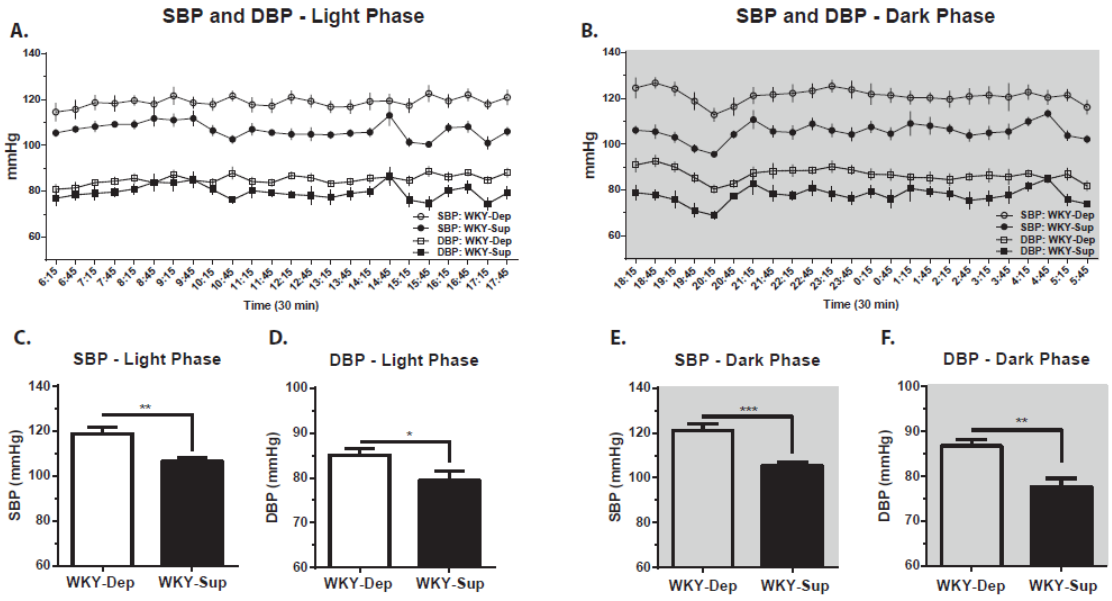

Figure 4.5. Methyl-supplementation lowers resting systolic blood pressure (SBP), and diastolic blood pressure (DBP). When 30-min average SBP values throughout light phase and overall average of SBP during light phase were compared between groups, WKY-Sup rats displayed lower SBP value throughout (A) and in overall average (C) compared to WKY-Dep rats. Similar results were observed during the dark phase, where WKY-Sup rats displayed lower SBP throughout (B) and in overall average (D). When 30-min average DBP values throughout light phase and overall average of DBP during light phase were compared between groups, WKY-Sup rats displayed lower DBP value throughout (A) and in overall average (E) compared to WKY-Dep rats. Similar results were observed during the dark phase, where WKY-Sup rats displayed lower SBP throughout (B) and in overall average (F). * - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$.

4.3.3. Stress-Evoked Cardiovascular Responses

Rats were briefly exposed to 10 minute novel rat (intruder) in their home-cage. Maximal HR and MAP response was determined by the maximal change in the value from baseline during the test period. In addition, curve-fitting analyses were performed to evaluate potential differences in HR and MAP post-stress recovery (Fig. 4.6A). No differences were detected in the maximal HR response:

WKY-Sup: 51.1±4.76 % vs. WKY-Dep: 36.27±6.96 % light ($t_{(12)} = 1.76$, $p = 0.10$; Fig. 4.6B). Curve-fitting analysis revealed significantly different curves that best represent HR recovery data between groups ($F_{3, 1688} = 14.28$, $p < 0.0001$; Fig. 4.6A). R^2 (i.e. goodness of fit index) for each curve was 0.33 and 0.49 for WKY-Dep and WKY-Sup groups, respectively, with the following estimated values: Y0 – 45.05 (WKY-Dep), 70.86 (WKY-Sup); Plateau – -1.94 (WKY-Dep), 0.88 (WKY-Sup), K – 0.045 (WKY-Dep), 0.076 (WKY-Sup). Data from individual rats were then curve fitted to estimate group differences in specific parameters of the recovery. This analysis revealed a significant increase of the decay constant in the WKY-Sup group ($t_{(12)} = 4.37$, $p = 0.0009$; Fig. 4.6C).

Analyses of the MAP data revealed no differences in the observed on maximal response to intruder ($t_{(12)} = 0.11$, $p = 0.92$; Fig. 4.7B). Curve-fitting of grouped data ($R^2 = 0.45$ WKY-Dep, $R^2 = 0.45$ WKY-Sup) showed a significant difference between groups ($F_{3,1688} = 4.268$, $p = 0.0052$; Fig. 4.7A) with the following estimated values: Y0 – 36.72 (WKY-Dep), 38.19 (WKY-Sup); Plateau – -2.49 (WKY-Dep), -0.31 (WKY-Sup), K – 0.055 (WKY-Dep), 0.073 (WKY-Sup) . Analyses of curve fits from individual animals revealed a significant increase of the decay constant in the WKY-Sup group ($t_{(12)} = 2.45$, $p = 0.03$; Fig. 4.7C).

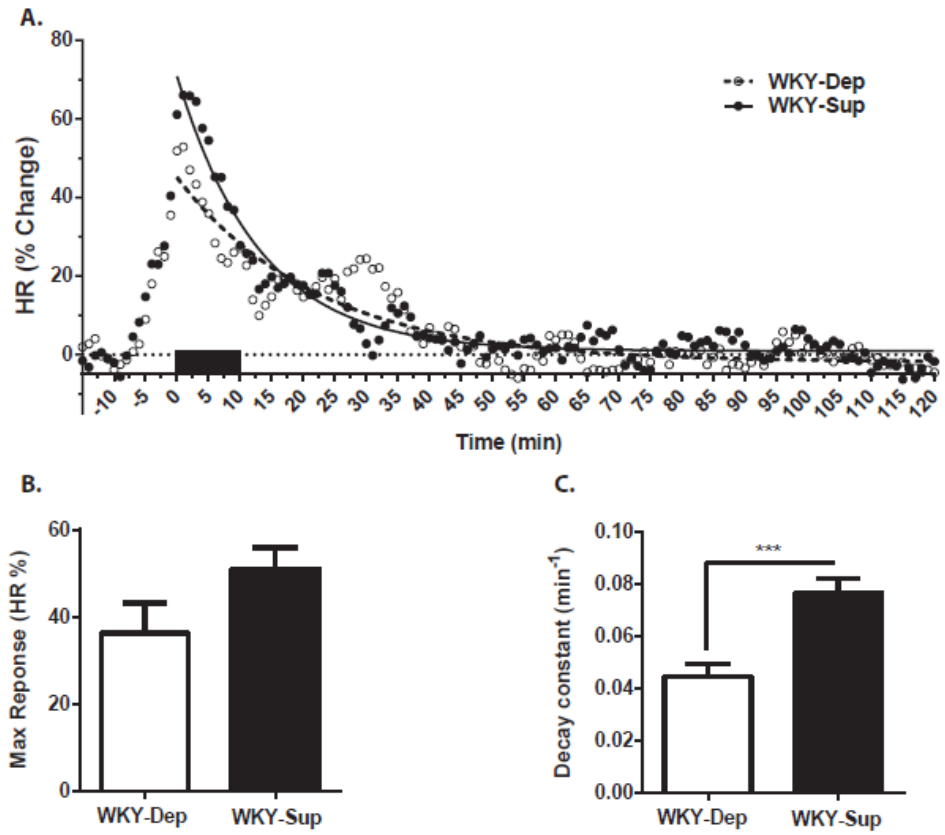

Figure 4.6. Heart rate (HR) response to intruder exposure. A novel age- and strain-matched intruder was exposed to the home cage of test rats for duration of 10 minutes. Maximal HR response was determined by the maximal change in the value from baseline (30 minute average value prior to 15 minute of the beginning of the test) during the test period. Curve-fitting analysis was performed on the percentage change (%change) value from baseline on HR data to investigate decay constant for CV recovery (A). No difference was observed on maximal HR response (B). However, WKY-Sup rats exhibited increased decay constant suggesting faster HR recovery (C). *** - $p < 0.001$.

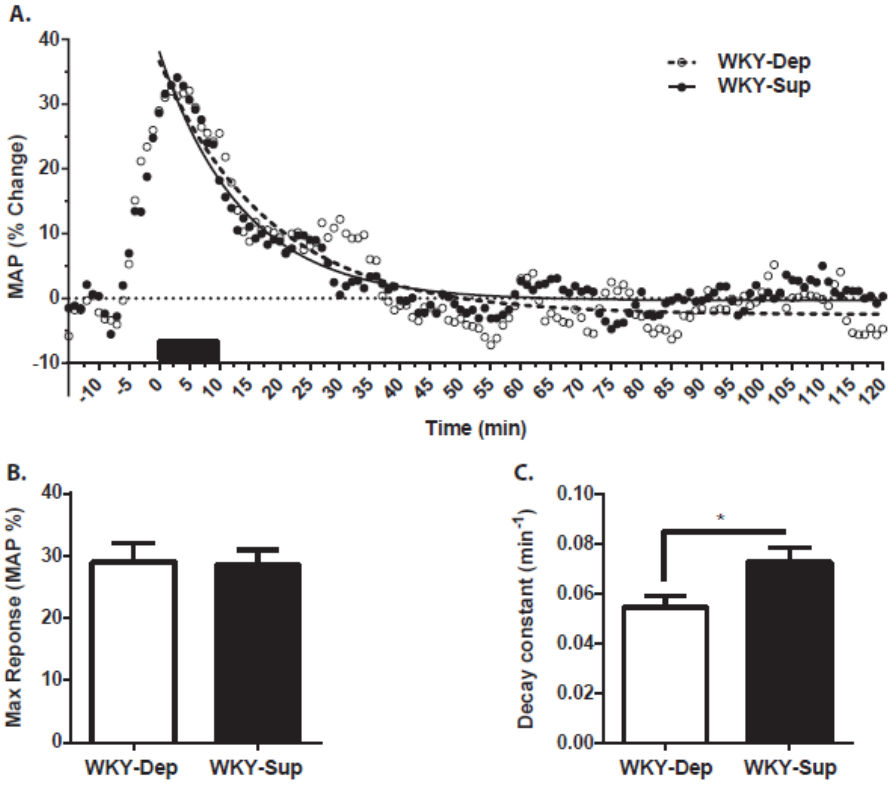

Figure 4.7. Mean Arterial Pressure (MAP) response to intruder exposure. A novel age- and strain-matched intruder was exposed to the home cage of test rats for duration of 10 minutes. Maximal MAP response was determined by the maximal change in the value from baseline (30 minute average value prior to 15 minute of the beginning of the test) during the test period. Curve-fitting analysis was performed on the percentage change (%change) value from baseline on MAP data to investigate decay constant for CV recovery (A). No difference was observed on maximal MAP response (B). However, WKY-Sup rats exhibited modest increase in decay constant suggesting faster MAP recovery (C). * - $p < 0.05$.

4.3.4. DNA Methylation and Methionine Levels

No difference was observed in the percent global methylation level in the hippocampus between the groups ($t_{(14)} = 0.57$, $p = 0.57$; Fig. 4.8A). However, WKY-Sup rats showed ~25% increase in the hippocampal content of methionine ($t_{(14)} = 3.74$, $p = 0.002$; Fig. 4.8B).

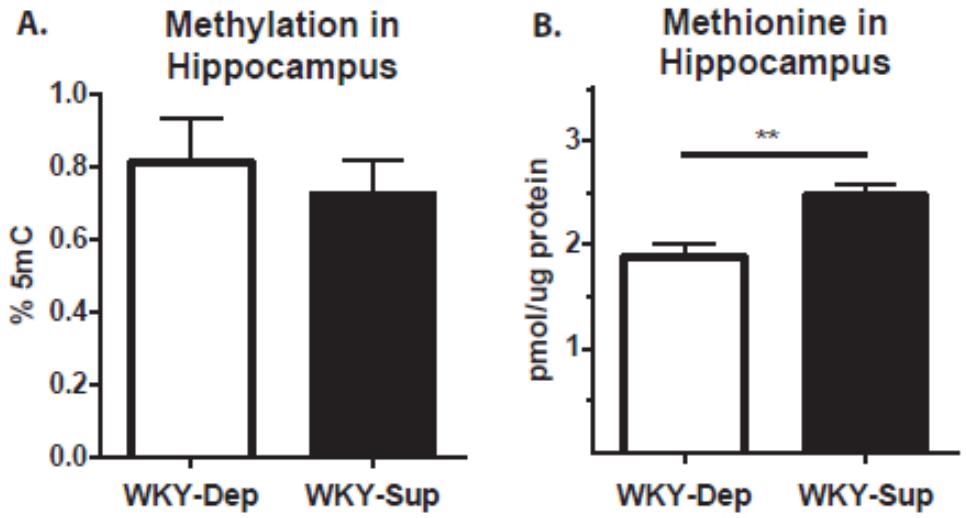

Figure 4.8. Whole genome methylation- and methionine- level in the hippocampus. Global methylation level and methionine levels were quantified in the hippocampus. No difference in global DNA methylation level in the hippocampus was observed between groups (A). However, increased level of methionine levels were observed in the hippocampus of WKY-Sup rats compared to WKY-Dep rats (B). ** - $p < 0.01$.

4.4 DISCUSSION

In the present study we report that diet-based methyl donor supplementation (vs. methyl donor depletion) leads to an increase in the exploratory behavior in the OFT, increased social interaction, and decreased immobility on the FST. Together these findings indicate a decrease in the anxiety- and depressive- like behaviors as a consequence of methyl donor supplementation. This manipulation also led to a decrease in the resting HR, increased HRV, and a decrease in resting blood pressure in the WKY-Sup rats. In addition to these baseline measures, methyl donor supplementation also improved cardiovascular recovery to social stress as evidenced by an increase in

the decay constants for HR and MAP following intruder exposure. Biochemical analyses confirmed an increase in the methionine levels in the hippocampus, but revealed no differences in the global DNA methylation in this brain region.

Previously, we found that maternal separation during early postnatal life can markedly increase the global DNA methylation levels in the hippocampus of adult WKY offspring. Thus, one of the goals of this study was to induce DNA hypermethylation via a diet-based approach to determine whether it would recapitulate behavioral and cardiovascular adaptations elicited by maternal separation. The results from the present study clearly show that manipulating the methyl-donor nutrients can induce adaptive behavioral and physiological changes in adulthood. Thus, although speculative, it is tempting to consider that the epigenetic programing through diet may open up new avenues for dietary interventions for mood and cardiovascular disorders.

It was surprising that we did not detect differences in global DNA methylation in the hippocampi of WKY-Sup and WKY-Dep rats. However, previous work demonstrated that depending on the organ, there is not always a direct relationship between methyl-donor levels in the diet and DNA methylation. For example, methyl-donor deficiency in the diet decreases global DNA methylation in the rat liver (Wainfan & Poirier, 1992), but not in the colon (Kim et al., 1995 ; Duthie et al., 2000). Global DNA methylation status is determined by the s-adenosyl methionine/s-adenosyl homocysteine ratio, which is critical for sustaining normal biochemical methylation reaction (Kim et al., 1995 ; Duthie et al., 2000). Moreover, global DNA methylation is also influenced by the levels and

activity of enzymes that regulate specific aspects of the DNA methylation process, such as DNA methyltransferases (McGowan et al., 2008). Therefore, multiple factors may explain our finding of the lack of DNA hypermethylation in the hippocampus.

WKY rats exhibit several behavioral features reminiscent of human depression, including increased anxiety- and depressive- like behavior along with social inhibition (Nam et al., 2014a). Behavioral inhibition in WKY rats is much greater than that in other rat strains, suggesting that it may not be possible to increase it further (Nam et al., 2014a). In the present study we did not compare effects of methyl donor depleted and methyl donor supplemented diets to a control diet. Thus, one question regarding our data is whether the observed effects are due to methyl donor supplementation or depletion. Given the WKY rats' high levels of behavioral inhibition, which are unlikely to be exacerbated, it is probable that the observed effects are due to methyl-donor supplementation because animals in the WKY-Sup group showed a decrease in their behavioral inhibition. However, studies that directly compare diet supplementation with methyl donors vs. non-supplemented diet will be necessary to directly address this issue.

In the present study we also show that methyl donor supplementation vs. depletion can significantly influence baseline cardiovascular indices, including HR, HRV and blood pressure. WKY-Sup rats exhibited decreased SBP (~14.0 mmHg lower), and DBP (~7.4 mmHg lower), which is in line with an earlier report showing that feeding methionine-enriched diet resulted in a decrease in MAP in

the Wistar-Hanover rats (Bjorvatn et al., 1995). Another group reported that although methionine-enriched diet increases SBP in Sprague-Dawley rats, it diminishes the development of hypertension in deoxycorticosterone acetate (DOCA)–salt hypertensive rats (Young et al., 1995). Another study reveal that methionine-supplemented diet increased SBP in normotensive WKY rats, but decreased SBP in the genetically related spontaneously hypertensive rats (Ursin, 1995). These studies have attributed these blood pressure changes to the levels of homocysteine, where hyper-homocysteine levels are associated with increased risk of cardiovascular (Ursin, 1995) as well as central nervous system dysfunction (Haug et al., 1995a). However, elevated levels of homocysteine results from high dietary methionine intake coupled with the deficiency of vitamin B12 and/or folate (Ursino et al., 1995), which is not case in the present study. While the levels of homocysteine and cysteine were below the levels of detection, we did not observe a difference in the concentration of cystine (data not shown), an amino acid that is synthesized from, cysteine suggesting that the observed effects were not due to differences in homocysteine. Interestingly, methionine supplementation in the diet elicits a decrease in novelty-induced locomotion in the Sprague-Dawley rats (Haug et al., 1995a), while we observed an increase in this behavior in WKY rats. This observation suggests that behavioral effects of methionine supplementation may be dependent on rat strain, and that similar to maternal separation WKY rats may respond to this intervention in a unique fashion.

Nutrition-induced epigenetic changes affect almost every organ and a variety of tissues. In the current study we focused on the hippocampus, a brain region that has been classically implicated in learning and memory along with the stress response. Past work from our group reported that early life experience differentially impacts behavior depending on the endogenous stress reactivity, so that WKY rats exhibit adaptive behavioral changes following maternal separation during their early postnatal period (Rana et al., 2015). In addition to these behavioral changes, maternal separation also elicits adaptive cardiovascular changes in the WKY rats that are accompanied by increased hippocampal global DNA methylation. Surprisingly feeding a methyl-donor enriched diet did not alter global DNA methylation in the hippocampus in the present study. However, we did observe an increase in the levels of methionine, which is a precursor of s-adenosyl methionine, a molecule that mediates DNA methylation (McGowan et al., 2008). Together these observations suggest that our diet manipulation may have induced differential methylation associated with specific genes. Future work utilizing gene-specific methylation analyses will be required to address this issue.

Several brain regions and their circuitry are implicated in regulation of behavior, stress-response, neuroendocrine, and autonomic responses. Besides contributing to decision-making, and memory formation, higher cognitive areas of the brain may also play roles in autonomic modulation and behavioral response to stress (Zamburlini et al., 1995). For example, forebrain regions such as prefrontal cortex (Wilhelmsen et al., 1995), bed nucleus of stria terminalis (Muller

et al., 1995), and hippocampus (Zamburlini et al., 1995) have been implicated in cardiovascular modulation and/or behavioral responses to stress. Hippocampal connectivity with the bed nucleus of stria terminalis, lateral septum, medial prefrontal cortex, and the amygdala has been reported in anatomical studies (Ursino & Cristalli, 1995a, b), suggesting that hippocampus may be at the center of integrating behavioral and cardiovascular responses to stress. It is feasible diet-based methyl donor supplementation or depletion elicits molecular alterations in the hippocampus that mediate the observed behavioral and cardiovascular adaptations.

Increases gene-specific DNA methylation in response to either s-adenosyl methionine or methionine treatment have been previously reported in the brain (Brage et al., 1995 ; Ursin et al., 1995). Furthermore, methionine infusion into the lateral ventricles has been shown to increase DNA methylation and the expression of the glucocorticoid receptor gene in the hippocampus (Haug et al., 1995b ; Weaver et al., 2005). Moreover, when gene expression changes were quantified in hippocampal tissue in methionine-treated rats, only 300 gene representing 1% of the gene population were affected, suggesting the specificity of the DNA methylation changes (Haug et al., 1995b). Thus, it has been suggested that epigenetic mechanisms are maintained through a complex interplay and dynamic equilibrium of enzymes (methylating and demethylating), which can be altered by specific agents involved in the epigenetic processes (McGowan et al., 2008).

CHAPTER 5: GENERAL DISCUSSION

5.1. Introduction

In the current study, we sought to investigate the programming effect of differences in early-life experience on behavior and baseline cardiovascular function. We also sought to determine the effects on DNA methylation induced by MS. Finally, we investigated the effects of diet-based manipulation of methyl-donor supplementation. We used WKY rat strain, a proposed rodent model of endogenous depression that also exhibits increased stress reactivity. The findings of these studies are: 1) behavioral effects of ELE seem to depend on endogenous stress reactivity of the rat strain, where WKY, but not Wistar, rats benefit from maternal separation (Chapter 2); 2) ELE also induced CV effects where maternally separated WKY rats exhibited protective cardiac indices (Chapter 3); 3) in contrast, trait aggression conferred adverse effects on blood pressure and vasculature independent of ELE (Chapter 3); 4) the behavioral and cardiovascular effects of MS were accompanied by increased hippocampal global DNA methylation (Chapter 4); 5) manipulating the methyl-donor content via the diet can recapitulate certain aspects of behavior and cardiovascular function observed in MS rats (Chapter 4). Taken together, data suggest that ELE influences behavior and cardiovascular function possibly through epigenetic mechanisms. Personality trait also had an effect on CV function independent of

ELE, possibly through different neurobiological mechanism which needs to be investigated further. Furthermore, dietary factors can have profound effect on behavior and CV function suggesting re-programming effect even after the post-weaning period.

5.2. Behavioral Effects of Maternal Separation

Early development represents a critical developmental period that is sensitive to environmental stimuli, and which regulate formation of specific brain circuits (Taylor, 2010). However, the effects of early life stress are not always adverse, suggesting the complexity in the trajectory of developing certain phenotypes during adulthood. The long-term biobehavioral effects on rats exposed to maternal separation have provided inconsistent results (Lehmann & Feldon, 2000), suggesting that the effects of maternal separation cannot be generalized. Our results show that depending on the rat strain and their endogenous stress reactivity, maternal separation can either be adaptive or maladaptive. Our research focus was on the rat strain (i.e. WKY rats) that successfully adapted to the early life stress resulting in beneficial behavioral as well as physiological outcomes. Although most of the current research focus on the vulnerability to early life stress, it is equally important to investigate the adaptation process and resiliency to early life stress. Evidence of a protective effect of early-life stress exposure comes from several human and animal studies. For example exposure to mild-to-moderate stressors during childhood seem to induce resilient phenotype or ‘stress inoculating’ effect, usually conferring beneficial advantage to subsequent stress exposure during adulthood

(Parker et al., 2006 ; Seery et al., 2010 ; Gapp et al., 2014). It has been suggested that the consequences of stress follow inverted U-shape curve where moderate stress exposure leads to health benefits (Seery et al., 2010 ; Chen & Miller, 2012). Research on non-human primates suggest that repeated stress exposure in childhood confers protective stress inoculation effect in cognition and emotional processing along with neuroendocrine adaptations (Lyons & Parker, 2007). The stress inoculating concept is consistent with the idea of a predictive adaptive response (PAR), which means that an individual will use experience of past stressors to augment coping with future stressors (Gluckman et al., 2007). Data demonstrating that rat pups exposed to the stress of either poor maternal care or 24-hour maternal deprivation exhibited increased memory performance and long-term potentiation as adults when tested under stressful conditions but not under non-stressful conditions supports the concept of PAR (Oomen et al., 2010). PAR is thought to be strongest in stress susceptible individuals and may be evolutionarily conserved by rapidly changing environmental conditions across generations (Gluckman et al., 2007). Our present data are consistent with this theory, showing that early-life MS has positive effects in the stress susceptible WKY rats. However, effect of MS on selectively bred rats that either display novelty-induced high locomotor activity (bHR) or novelty-induced low locomotor activity (bLR) shows that adult bHR offspring are found to be resilient whereas adult bLR offspring are found to exhibit increased stress induced defecation and corticosterone response (Clinton et al., 2014). Thus, the speculation that stress susceptible individuals have pronounced PAR cannot be entirely through the

stress responsivity profile. Other factors such as differences in the strain, biobehavioral profile, and coping ability might explain the differences between divergent effect of MS in bLRs and WKY rats. Further investigations are needed to explain the divergent effect of MS between strains.

5.3. Cardiovascular Effect of Maternal Separation

Several studies have tried to address the effects of early-life stress on cardiovascular function. For example MS has been shown to have mild effect on cardiac autonomic balance and heart structure during adulthood (Trombini et al., 2012). In a rat strain that is sensitive to salt induced hypertension, MS induced impaired blood pressure recovery when exposed to acute stress, with no differences in baseline blood pressure (Loria et al., 2015). Studies in WKY rats showed that chronic infusion of angiotensin sensitizes hypertensive response along with vascular inflammation and renal dysfunction MS exposed rats (Loria et al., 2010b ; Loria et al., 2013). However, no difference in baseline cardiac parameters was observed. The discrepancies between these studies and the current study may be due to the differences in MS protocol itself where Loria et al. expose half of the litter to MS whereas the other half litter serve as unseparated controls (Loria et al., 2010b ; Loria et al., 2013). In the present study we separated the whole litter for either 180 min (MS) or 15 min (NH). The differences in MS protocol itself may induce differences in maternal behavior, which may differentially impact function in adulthood. Furthermore, no behavior parameters were analyzed in the above mentioned studies compared to the current study.

In the present study protective behavioral effects of MS in WKY rats were accompanied by lower resting HR, increased HRV, and increased sBRS throughout the lifespan (P133 through P277). These effects indicate that MS may confer adaptive behavioral and physiological changes, which is supported by previous work. For example, early-life stress induced by limited access to nesting and bedding during the early postnatal period conferred protective metabolic consequences in adulthood in response to a high fat/high sugar diet (Maniam et al., 2015). In adolescent/young adult individuals that experienced parental loss during their childhood, emotional and cardiovascular adaptation was observed in the presence of high perceived caring from surviving parent (Luecken et al., 2009). Furthermore, Luecken et.al., reported that the effect of early-life stress induced by childhood parental loss can lead to either maladaptation (stress sensitization) or adaptation (stress inoculation) in terms of emotional and cardiovascular system which might be mediated by survival parental care (Luecken et al., 2009).

5.4. Brain Circuits that Regulate Stress Responses and Cardiovascular Function

Central nervous system plays a prominent role in cardiovascular regulation. Pre-autonomic sites in the brainstem (i.e. rostral ventrolateral medulla [RVLM] and nucleus tractus solitarius [NTS]) and hypothalamus (i.e.paraventricular nucleus [PVN]) provide direct inputs to autonomic efferents (Amendt et al., 1979 ; Cox & Brody, 1989a, b ; Oparil et al., 1989 ; Badoer et al., 1993 ; Dampney, 1994 ; Sved & Ruggiero, 1996 ; Waldrop et al., 1996 ; Coote et

al., 1998 ; Verberne & Owens, 1998 ; Guyenet, 2006 ; Bathina et al., 2013 ; Totola et al., 2013 ; Jiang et al., 2014 ; Kawabe et al., 2014). Higher-order limbic regions, including the medial prefrontal cortex (mPFC), amygdala, the bed nucleus of stria terminalis (BNST) and the septum, are engaged by emotional stressors and impact cardiovascular function via their projections to the pre-autonomic sites (Jordan, 1990 ; Verberne et al., 1997 ; Verberne & Owens, 1998). Hippocampus (HPC) plays a central role in the regulation of social behaviors (Kimble, 1968 ; Wallace et al., 1977 ; Sams-Dodd et al., 1997 ; Becker et al., 1999) and sends dense projections to mPFC, amygdala and septum (Krettek & Price, 1977 ; Swanson & Cowan, 1977 ; Goldman-Rakic et al., 1984 ; Henke, 1990 ; Jay & Witter, 1991 ; Barbas & Blatt, 1995 ; Verwer et al., 1997 ; Pitkanen et al., 2000 ; Petrovich et al., 2001). Its multi-synaptic projections reach the NTS via a relay in mPFC(Swanson, 1977, 1981 ; Ruit & Neafsey, 1990 ; Canteras & Swanson, 1992 ; Verberne & Owens, 1998), and PVN by the way of BNST(Herman & Cullinan, 1997 ; Herman & Mueller, 2006). Recordings in humans showed that firing of nearly one third of HPC neurons is modulated by the cardiovascular cycle, and that their baseline firing correlates with the resting heart rate (Terreberry & Neafsey, 1987 ; Frysinger & Harper, 1989). Electrical and chemical stimulation of HPC lowers resting HR and MAP in a variety of species (Smith, 1944 ; Kaada, 1951 ; Anand & Dua, 1956 ; Ruit & Neafsey, 1988) and can also inhibit peristalsis and alter pupillary dilatation (Carlson et al., 1941 ; Kaada, 1951 ; Andy & Akert, 1953). These data suggest that HPC is uniquely positioned to integrate behavioral and cardiovascular responses to

social stress. Thus, HPC could possibly be a key region to investigate the behavioral and cardiovascular alterations induced by ELE.

5.5. Early Environment and the Epigenome

Epigenetic mechanisms lie at the crossroads where nature meets nurture and mediate gene X environment interactions implicated in disease emergence (Caspi et al., 2003 ; Petronis, 2010 ; Portela & Esteller, 2010 ; Hallmayer et al., 2011), including cardiovascular disorders (Handy et al., 2011 ; Lorenzen et al., 2012 ; Turunen et al., 2013 ; Aslibekyan et al., 2014 ; Chaturvedi & Tyagi, 2014). DNA methylation at CpG islands is one of the most well-known epigenetic mechanisms, and it results in enduring changes in gene expression (Szyf et al., 2005 ; Mueller & Bale, 2008 ; Zhang et al., 2010 ; Mychasiuk et al., 2011). The genome is comprised of a billion cytosines that are dynamically methylated throughout neurodevelopment, aging, learning, and following stress. Environmental factors including life experience modify epigenetic processes in a variety of tissues, including the brain (McGowan et al., 2008 ; McGowan et al., 2009 ; Murgatroyd et al., 2009 ; Roth et al., 2009 ; LaSalle, 2011 ; Franklin et al., 2012 ; Naumova et al., 2012). Thus in the present study, we focused on ELE induced alteration in global DNA methylation in brain regions implicated in emotional, behavioral, and cardiovascular functioning. Of the brain regions examined, hippocampal global DNA hypermethylation was observed in MS rats, suggesting the specificity of epigenetic alterations. Hippocampus has been implicated in cardiovascular modulation and behavioral responses to stress

(Zamburlini et al., 1995). Hippocampal connection to other brain regions such as bed nucleus of stria terminalis, lateral septal area, medial prefrontal cortex, and amygdala has been reported in anatomical studies (Petrovich et al., 2001), (Swanson & Cowan, 1977). Given the connectivity of the hippocampus with several brain structures, which modulate behavior and cardiovascular function, it may be a key structure in integrating behavioral and cardiovascular functions.

5.6. Personality Traits

Evolutionary traits such as aggressive behaviors are required for survival in the face of competition from rivals for territory, food, mates and for self-preservation from predator threats (Natarajan & Caramaschi, 2010 ; Lindenfors & Tullberg, 2011). However, excessive aggression can be pathological and is associated with a host of emotional, neurobiological, and cardiovascular abnormalities. On the other hand, aggressive behaviors induced by social conflict can be reflective of an animal's coping strategy to successfully adapt to certain situations.

In the present study, WKY rats exhibited either attack behavior or no-attack behavior in response to intruder exposure that was found to be independent of ELE. The observed attack behavior reflects the individual differences of personality traits. On baseline parameters, rats that attacked the intruder exhibited increased resting blood pressure, increased aortic response to phenylephrine, increased wall-to-lumen ratio, and increased norepinephrine content in the left ventricle in comparison to the rats that did not attack the

intruder. These data suggest adverse cardiovascular changes are associated with trait aggression.

5.7. Conclusions and Future Directions

Our results demonstrate that maternal separation does not necessarily lead to negative consequences, but may also confer adaptive changes. These results are important in highlighting the adaptation process induced by MS and producing protective behavioral as well as cardiovascular changes. Furthermore, the current study also demonstrates the impact of diet supplements on behavior and cardiovascular system concurrently, which is potentially mediated via epigenetic changes. The results from the diet manipulation study are promising for translational research where supplementing diet with various methyl donors may produce beneficial behavioral and cardiovascular changes.

In future studies, it would be interesting to find the relationship between maternal care and the resulting behavior and CV parameters along with DNA methylation profile. Several investigations have reported that naturally occurring differences in maternal care profoundly affect the behavior and epigenetic alteration in the offspring (Caldji et al., 1998 ; Francis et al., 1999 ; Meaney & Szyf, 2005). Other studies have reported that maternal separation or maternal diet can influence the cardiovascular system in rodents (Trombini et al., 2012 ; Maniam et al., 2015). However, the role of maternal care in rodent offspring's cardiovascular function is largely unkown. Thus, future studies will be needed to elucidate the interplay among maternal care, offspring behavior, cardiovascular

function, and epigenetic changes.

Additionally, we would like to examine gene-specific alteration in DNA methylation of the hippocampus. In the current study, we found that maternal separation led to increased global DNA methylation in the hippocampal DNA and supplementing methyl-donor supplements in diet led to increased methionine content in the hippocampus (Chapter 4). We would like to extend this observation by utilizing next-generation sequencing with methylated DNA capture (MethylCap-seq) (Bock et al., 2010 ; Brinkman et al., 2010 ; Gu et al., 2010 ; Aberg et al., 2012 ; Parrish et al., 2012 ; Day et al., 2013) to examine methylated regions throughout the genome rather than focusing solely on gene promoter-associated CpGs (a limitation of alternative methodologies)(Harris et al., 2010 ; Laird, 2010 ; Aberg et al., 2013). Although increased global DNA methylation and/or increased methionine content suggest the involvement of epigenetic changes, this method does not capture the gene-specific alteration. Thus, the investigation on gene-specific changes will provide information about the epigenetically altered genes that are potentially responsible for the alteration in behavior and CV function.

Another approach to confirm the role of hippocampus in mediating behavior and CV function is to inject methyl donors, which enhance DNA methylation, in the hippocampus and study the resulting phenotypes. Using intracranial injection technique to deliver the methyl-donor drug into hippocampus will preclude the possibility of secondary effects produced by possible alterations in DNA methylation profile in other organs and tissue induced by diet

manipulation.

Overall, we would like to determine a mechanistic link between ELE and the behavioral and CV outcome during the adulthood and how epigenetic mechanisms such as DNA methylation in the hippocampus play a role in mediating those effects. Although the involvement of other brain regions and the neural circuit that have connection to the hippocampus are taken into the consideration, we suspect that hippocampus is one of the key regions that might be involved in mediating behavioral and CV regulation concurrently.

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APPENDIX

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: October 11, 2012

TO: ILAN KERMAN, M.D.
SC -7TH 0017
FAX:

FROM: Judith A. Kapp
Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: **NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.**

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on October 11, 2012.

Title of Application: Effects of Maternal Separation and Neonatal Handling on Cardiovascular Function
Fund Source: Internal

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)

Institutional Animal Care and Use Committee
CH19 Suite 403
933 19th Street South
205.934.7692
FAX 205.934.1188

Mailing Address:
CH19 Suite 403
1530 3RD AVE S
BIRMINGHAM AL 35294-0019

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: December 19, 2012

TO: ILAN KERMAN, M.D.
SC -7TH 0017
FAX:

FROM: Judith A. Kapp
Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: **NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.**

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on December 19, 2012.

Title of Application: Emotional Behavior and Early Life Stress in a Rat Model of Depression

Fund Source: Internal

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)

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BIRMINGHAM AL 35294-0019

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: September 6, 2013

TO: ILAN KERMAN, M.D.
SC -7TH
(205) 975-0310

FROM:

Robert A. Kesterson, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: **NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.**

The following application was renewed by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on September 6, 2013.

Title: Effects of Maternal Separation and Neonatal Handling on Cardiovascular Function
Sponsor: Internal

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

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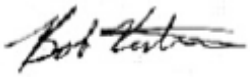
THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: October 14, 2013

TO: ILAN KERMAN, M.D.
SC -7TH
(205) 975-0310

FROM: 

Robert A. Kesterson, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: **NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.**

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on September 19, 2013.

Title: Impact of Chronic Stress on Brain Circuits Regulating Behavior and Cardiovascular Function (Rana)
Sponsor: American Heart Association (Southeast Affiliate)

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Institutional Animal Care and Use Committee (IACUC)
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THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: March 10, 2014

TO: ILAN KERMAN, M.D.
SC -7TH
(205) 975-0310

FROM: 

Robert A. Kesterson, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: **NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.**

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on March 10, 2014.

Title: Neural Substrates of Somatomotor and Autonomic Disturbances in Major Depression
Sponsor: NIH

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

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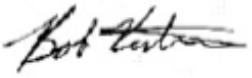
THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: September 25, 2014

TO: ILAN KERMAN, M.D.
SC -7TH
(205) 975-0310

FROM: 

Robert A. Kesterson, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: **NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.**

The following application was renewed by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on September 25, 2014.

Title: Impact of Chronic Stress on Brain Circuits Regulating Behavior and Cardiovascular Function (Rana)
Sponsor: American Heart Association (Southeast Affiliate)

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

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