Neonatal maternal separation stress elicits lasting DNA methylation changes in the hippocampus of stress-reactive Wistar Kyoto rats

Running title: Early-life stress, methylation, and hippocampus

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The proposed Journal Section for this manuscript is: Behavioral Neuroscience & Clinical and Translational Neuroscience (Section Editor, Dr. James Herman). The manuscript contains 9105 words in the entire manuscript, 249 words in the Abstract, and 484 words in the Introduction.

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Keywords
Depression, Anxiety, Epigenetics, Folate, Methyl Depletion, Resilience

Abstract

Early-life stress (ELS) can alter neurodevelopment in variable ways, ranging from producing deleterious outcomes to stress resilience. While most ELS studies focus on its harmful effects, recent work by our lab and others shows that ELS elicits positive effects in certain individuals. We exposed Wistar-Kyoto (WKY) rats, known for a stress reactive, anxiety-/depression-like phenotype, to maternal separation (MS), a model of ELS. MS exposure elicited anxiolytic and antidepressant behavioral effects as well as improved cardiovascular function in adult WKY offspring. The present study interrogates an epigenetic mechanism (DNA methylation) that may confer the adaptive effects of MS in WKY offspring. We quantified global genome methylation levels in limbic brain regions of adult WKYs exposed to daily 180-min MS or neonatal handling from postnatal day 1-14. MS exposure triggered dramatic DNA hypermethylation specifically in the hippocampus. Next-generation sequencing methylome profiling revealed reduced methylation at intragenic sites within two key nodes of insulin signaling pathways: the insulin receptor and one of its major downstream targets, mitogen activated protein kinase kinase kinase 5 (*Map3k5*). We then tested the hypothesis that enhancing DNA methylation in WKY rats would elicit adaptive changes akin to the effects of MS. Dietary methyl donor supplementation improved WKY rats' anxiety/depression-like behaviors and also improved cardiovascular measures, similar to previous observations following MS. Overall these data suggest a potential molecular mechanism that mediates a predicted adaptive response whereby ELS induces DNA methylation changes in the brain that may contribute to successful stress coping and adaptive physiological changes in adulthood.

Introduction

Adverse early-life experiences have long-term effects on the brain and biobehavioral responses to stress in many species (Suomi, 1991; Daniels *et al.*, 2004; Rensel *et al.*, 2010; Thompson *et al.*, 2012). Although most work on ELS highlights its harmful effects such as increasing risk for neuropsychiatric disorders (Hofer *et al.*, 1996; Sanchez *et al.*, 2001; Maccari *et al.*, 2014), smoking, physical inactivity, obesity, and cardiovascular disease in humans (Dong *et al.*, 2004), emerging evidence suggests a more nuanced view. The match/mismatch hypothesis of disease states that ELS can have adaptive value to individuals facing later stressful experiences (Santarelli *et al.*, 2014). For instance, repeated childhood stress exposure confers protective neuroendocrine effects when adult offspring face stress (Lyons & Parker, 2007). This suggests that repeated ELS elicits a predictive adaptive response (PAR) where past stressful experiences augment coping with future stress (Gluckman *et al.*, 2007). PAR is thought to be strongest in stress-susceptible individuals and may be evolutionarily conserved by rapid cross-generational environmental changes (Gluckman *et al.*, 2007; van der Doelen *et al.*, 2013). Our recent work lends further support to this theory. We exposed Wistar-Kyoto (WKY) offspring, a stress susceptible rat strain that exhibits behavioral abnormalities commonly associated with depression and anxiety disorders (Nam *et al.*, 2014), to daily 3-hour maternal separation (MS-180) during the first two weeks of life. While MS-180 has frequently been used to demonstrate deleterious effects on adult rodent emotional behavior [for review see (Sanchez *et al.*, 2001)], we found that MS-180 elicits several adaptive effects in adult WKY offspring, including diminished behavioral despair, increased social exploration (Rana *et al.*, 2015), and improved cardiovascular measures, such as decreased baseline heart rate (HR) and increased HR variability (Rana *et al.*, 2016).

The present study exploited the WKY-MS model to identify neurobiological and epigenetic mechanisms that mediate a PAR to convey adaptive behavioral and physiological

changes in adult MS-180-exposed WKY offspring. We hypothesized that MS-180 triggers DNA methylation changes in the brains of WKY offspring, which may contribute to their enhanced stress resilience in adulthood (Rana *et al.*, 2015). To test this, we first examined global DNA methylation (5-methylcytosine) levels in multiple brain regions of adult WKY rats that were exposed to MS-180 (or control condition). Because we found dramatic MS-180-induced hypermethylation in the WKY hippocampus, we then used next-generation sequencing to interrogate gene-specific methylation changes that occurred following MS-180 exposure. Our final experiment tested the hypothesis that enhancing DNA methylation in adult WKYs (by increasing dietary methyl donor content) would elicit adaptive behavioral and cardiovascular changes akin to what occurred following MS-180 exposure. Specifically, we predicted that feeding adult male WKYs a methyl-donor fortified diet would: *(a)* have anxiolytic and antidepressant effects in tests of anxiety- and depression-like behavior; and *(b)* elicit positive cardiovascular changes, including reduced baseline HR and greater HR variability, similar to what we previously observed in MS-180-exposed WKY offspring (Rana *et al.*, 2015; Rana *et al.*, 2016).

Materials and Methods

All experiments were approved by the Committee on the Use and Care of Animals at the University of Alabama at Birmingham. This work was performed in accordance with the National Institutes of Health (USA, 2011) and National Research Council (UK, 1996) guidelines on animal research.

Animals

Adult male and female WKY rats (n = 8/sex) 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.). For the first phase of the experiment, male/female pairs were mated for 14 days, and at birth (postnatal day 0) litters were randomly assigned to one of two groups: (a) MS-180 group or (b) neonatal handling (NH) group (n = 4 litters/group/strain). Litters assigned to the MS-180 and NH groups were separated from their dam daily for 180 min and 15 min, respectively, between 8:30 a.m. – 12:00 p.m. from postnatal day (P)1-P14 as previously described (Clinton *et al.*, 2014; Rana *et al.*, 2015). 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- (NH) or 180- (MS-180) min period. After the final separation on P14, litters remained undisturbed until weaning on P21. Afterwards, male rats of the same early-life experience (NH or MS-180) were housed together three per cage, and a subset of these animals (n = 10 NH, n = 10 MS-180) were utilized in subsequent behavioral and physiological studies that have been reported previously (Rana *et al.*, 2015; Rana *et al.*, 2016). Details of the behavioral and physiological studies can be found in these reports. The animals were euthanized during postnatal weeks 49-50, and their tissue was used for molecular studies reported here.

The second experiment manipulated dietary methyl donor content in a separate group of adult male WKY rats (n = 28) that was purchased from Charles River Laboratories (Kingston, NY). Upon arrival, rats were housed two per cage of the in our animal housing facility. Following a one-week acclimatization period, rats were assigned to either a diet depleted of (n = 14) or supplemented with (n = 14) methyl donors (see below). After four weeks of receiving these modified diets, rats were evaluated on a behavioral test battery and later instrumented with radiotelemetry probes (PA-C40, DSI International; n = 8 per group) for cardiovascular assessments (procedural details in subsequent sections).

Assessing global DNA methylation (5-methylcytosine) levels in MS-exposed WKY brain

We examined global DNA methylation (5-methylcytosine) levels in several brain regions of adult MS-180- and NH- exposed male WKY offspring. Brains were removed, flash frozen in isopentane cooled to -30°C on dry ice, and then stored at -80°C. Brains were sectioned on a cryostat at -10 to -12°C, and alternating sections of 20 and 300 µm were collected. The 20 µm sections were stained with cresyl violet to identify target anatomical regions in the 300 µm sections. Portions of the hippocampus, paraventricular nucleus of the hypothalamus (PVN), amygdala, septum, bed nucleus of the stria terminalis (BNST), and the medial prefrontal cortex (mPFC) were removed from the 300 µm-thick sections using a 0.5 mm tissue punch (Harris Micro-Punch, Ted Pella, Redding, CA). One group of samples was used to isolate DNA (DNeasy, Qiagen, Hilden, Germany; n=10/group), which was later quantified using the Nanodrop ND-1000 (Wilmington, DE), and stored at -20°C. Due to the small volume of tissue collected from PVN, these samples were pooled in pairs yielding an experimental sample size of five per group. We assessed levels of 5-methylcytosine (an indicator of DNA methylation) using the Epigentek MethylFlash Methylated DNA Quantification Kit (Colorimetric) per manufacturer's instructions as described (Simmons *et al.*, 2013). Briefly, 200 ng of genomic DNA was loaded per well and the samples were run in triplicate. Sample values were compared to a standard curve and to calculate the percent of methylated DNA as described in the manufacturer's protocol.

Methylated DNA capture coupled with next-generation sequencing (MethylCap-Seq) in hippocampus of MS-exposed WKYs

To obtain a more detailed understanding of DNA methylation differences in the adult hippocampus of MS-180- vs. NH- exposed WKY offspring, we performed a MethylCap-Seq experiment. Methylated DNA was captured using a MethylMiner DNA enrichment kit (Applied Biosystems, Grand Island, NY, ME10025) according to the manufacturer's recommended

protocol. Adult MS-180/NH WKY hippocampus DNA samples (n = 4/group) were sheared by sonication using a Bioruptor Pico device (Diagenode, Denville, NJ, B01060001). The sonication cycle consisted of 15 s on-45 s off for seven total cycles to generate fragments of (on average) 325 base pairs for the methylated DNA enrichment protocol (Brinkman *et al.*, 2010). Methylated DNA fragments were captured with a methyl-binding domain 2 (MBD2) protein coupled to paramagnetic Dynabeads® M-280 Streptavidin per manufacturer instructions and as described (Brinkman *et al.*, 2010). Starting material for the enrichment protocol was 1µg of sonicated DNA, and captured fragments were eluted using 3,500 mM NaCl. Captured material was purified using QIAquick PCR purification spin columns (Qiagen, Valencia, CA); it was then quantified using Quant-it high sensitivity DNA Assay Kit (Invitrogen, Grand Island, NY, Q-33120) and the Agilent 2100 Bioanalyzer high sensitivity chip kit (Agilent Technologies, Santa Clara, CA). Samples were sent to HudsonAlpha Genomic Services Laboratory (Huntsville, AL; http://gsl.hudsonalpha.org) for next-generation sequencing using NEBNext reagents (New England Biolabs, Ipswich, MA) according to manufacturer's recommendations. Barcoded DNA fragment libraries were created, checked for quality, and quantified with the Kapa Library Quant Kit (Kapa Biosystems, Wilmington, MA). Afterwards, each library was used for high-throughput sequencing on an Illumina HiSeq2000 (Illumina, San Diego, CA) with 25M total 50 base pair single-end reads per sample. Up to six barcoded samples were loaded per lane of a flowcell and sequenced using a paired-end 50-base pair protocol (according to manufacturer's recommendations). We sequenced four biological replicates per group (with each derived from unique litters) as well as an input (non-captured) control for normalization.

To ensure that MBD2 protein capture resulted in specific enrichment of methylated DNA, we performed control reactions. In these reactions, gDNA was spiked with synthetic methylated and non-methylated DNA fragments (1 pg each, Methyl Miner kit, Invitrogen, Grand Island, NY) prior to immunoprecipitation with recombinant MBD2. We performed PCR with primers for these synthetic fragments within the methylated and non-methylated DNA capture, using both

the captured (MBD2-bound) and unbound fractions. Our results demonstrated the presence of methylated DNA fragments in the captured sample, and absence of methylated DNA in the unbound fraction.

After next-generation sequencing was complete, we imported data files into Galaxy (https://usegalaxy.org/), an online data analysis system that facilitates large-scale genome analyses (Giardine *et al.*, 2005; Blankenberg *et al.*, 2010; Goecks *et al.*, 2010). Raw single-end sequenced reads were quality controlled and filtered for read quality (FastQC, Galaxy). Sample reads were mapped onto the rat genome reference sequence (Rn5 assembly) using the high-performance alignment software Bowtie for Illumina (http://bowtie-bio.sourceforge.net/index.shtml). Overall we obtained an average of 27M mapped single-end reads from Methyl-Cap samples. Genome-aligned sequenced reads were examined using SeqMonk (Babraham Institute; www.bioinformatics.babraham.ac.uk/projects/seqmonk) and methylation levels were assessed via built-in analysis pipelines. We used Model-based Analysis of ChIP-Seq (MACS) for methylated peak detection through comparison to input and enriched samples and applying a p-value of 0.05 (Zhang *et al.*, 2008). We then performed read count quantitation with reads per million and probe length corrections. Data were visualized using a Manhattan plot via qqman, an R package (Turner, 2014). Statistical analysis compared intensity differences between WKY-MS-180 and WKY-NH samples (p-value of 0.1 with Benjamini-Hochberg (BH) multiple test corrections) across the genome to evaluated distinct MS-180 vs. NH methylation patterns within several genomic features, including: CpG islands (GC content ≥50%, length >200 base pairs); exons and introns within specific genes; and potential gene promoters (2 kb upstream from 5′ transcription start sites (TSS). Where applicable, methylation level is described in normalized read counts, which were normalized by reads per million and by the size of methylated peak (probe length). We performed Gene Ontology (GO) analysis for biological processes through WebGestalt (http://bioinfo.vanderbilt.edu/webgestalt/) with genes that have differentially methylated regions within the gene body or within 2 kb

upstream of the TSS.

Quantitative real-time PCR confirmation of select genes showing differential methylation in hippocampus of MS-exposed WKY rats

In order to confirm that MS-180-related DNA methylation differences in the hippocampus lead to predicted mRNA expression changes within select genes, we conducted quantitative Real Time PCR (qRT-PCR) using a StepOne Plus (Applied Biosystems, Grand Island, NY, USA) with TaqMan detection chemistry as described by manufacturer. We examined the expression of the following genes: *Insr* (Rn00690703_m1; RefSeq: NM_017071), *Map3k5* (Rn01440422_m1; RefSeq: NM_001277694.1), *Igf1r* (Rn00583837_m1; RefSeq: NM_052807.2) and *Grik4* (Rn00561331_m1; NM_012572) and used *Abl1* (Rn01436239_m1; NM_001100850) as a housekeeping gene. RNA was isolated (NucleoSpin RNA II) from hippocampal samples collected from adult WKY rats that were exposed to MS-180 or control condition in early life (n = 5 per condition, with each derived from unique litters). We reverse transcribed 100 ng of RNA to cDNA using Invitrogen SuperScript VILO (Invitrogen, Grand Island, NY). Reactions were carried out in 96-well qPCR plates (Qiagen), with each well loaded with 20 µl of a mix of cDNA, TaqMan qPCR mastermix (ThermoFisher Scientific), RNase-free water, and TaqMan primers. The PCR began with a two-min. hold at 50°C and then Taq Polymerase was activated by heating the plate to 95°C for ten min. Amplifications were performed for 40 cycles, each one consisting of 95°C for 15 s and 60°C for one min. Amplifications of all samples were carried out in triplicate, and the average cycle threshold (CT) was calculated as a geometric mean for each sample. Relative fold changes between WKY-MS-180/NH groups were compared for a given gene at a particular time point were calculated using the ΔΔCT method.

Dietary methyl donor manipulation, behavioral and physiological assessment in adult WKY rats

In order to begin to assess the functional implications of MS-180-induced DNA methylation changes in WKY offspring, we conducted an experiment to test whether inducing DNA hypermethylation in the adult WKY rat brain mimics behavioral and cardiovascular effects akin to what we observed in adult offspring with a history of early-life MS (Rana *et al.*, 2015). To do so, we manipulated methyl donor content in the diet of adult WKY males by feeding them either a diet that was depleted of methyl donors or a diet supplemented with high levels of methyl donors. Adult male WKY rats purchased from Charles River were allowed to acclimate to our housing facilities for one week before being randomly assigned to receive either: *(1)* chow that was 90% depleted of methyl donors (WKY-DEP); or *(2)* a methyl supplemented diet (WKY-SUP; n = 14 WKY males per group). The WKY-DEP group received a rodent diet lacking 90% of normal requirements of choline, folate, and methionine (diet no. A04062402, Research Diets Inc, New Brunkswick, NJ), and the WKY-SUP group received chow fortified with increased amounts of cofactors and methyl donors (folic acid, choline, methionine, and Vitamin B12; diet. No. A04062403, Research Diets Inc, New Brunkswick, NJ; **Table 1**). The diets otherwise were matched for their overall caloric content and their L-amino acid content (except for L-methionine). Animals within both diet groups were matched for their caloric intake (Konycheva *et al.*, 2011; Ishii *et al.*, 2014) to receive 35 g of chow/cage/day during the first three weeks, and then increased to 40 g of chow/cage/day during the remaining 7 weeks of the experiment when behavioral testing and later cardiovascular assessments were conducted. Each cage housed two rats. Animals were weighed each week starting on the first day of diet exposure.

Behavioral test battery: After receiving the methyl-depleted or methyl-supplemented diets for four weeks, rats were evaluated in a behavioral test battery comprised of several classic rodent tests of anxiety- and depression-related behavior: *a)* Open Field Test; *b)* a Social Interaction test; and *c)* the Forced Swim Test (FST). All rats were subjected to the full test battery in this test order, with 1-2 days' rest between tests. Tests were performed between 8:00 a.m. – 12:30

p.m. and conducted under dim lighting (30 lux).

The open field test was conducted in a Plexiglas white box (100×100×50 cm) with a black floor. At the beginning of the test, a rat was placed into the corner of the box and was permitted to explore the apparatus for 5 min. The latency to enter the center of the open field, the amount of time spent and distance traveled in the center, periphery, and corners of the apparatus were quantified utilizing Ethovision® XT 8.0 videotracking software (Noldus, Wageningen, The Netherlands) set up with a digital video camera. A trained observer, who was blinded to experimental groups, manually assessed grooming and rearing behavior using a computerized system provided in the software.

The social interaction test was performed in a black Plexiglas box (30 x 90 x 60 cm) that was divided into three chambers (zones) separated by two black Plexiglas dividers with openings in the center to allow experimental rats to move freely between zones. The test was comprised a single 10-min session where the test rat was placed in the neutral zone (middle chamber), while one of the adjacent chambers contained a novel object (empty cage) and the third chamber contained a novel male stimulus rat within metal cylindrical interaction cage. The metal bars of the interaction cage allowed rats to interact, but prevented any aggressive encounters between animals. Stimulus male rats were age-matched and of the same strain as the test animals, and were previously habituated to interaction cages. Rats' behavior was videotaped, and Ethovision® XT 8.0 videotracking software (Noldus, Wageningen, The Netherlands) was used to analyze the latency for experimental animals to enter chambers containing social stimulus rats as well as the number of visits and time spent in each chamber.

Porsolt's FST was performed as we previously described (Nam *et al.*, 2014) with 30-cm deep 25°C water in Plexiglas containers (45 cm high x 20 cm diameter). On FST day 1, rats were placed (1/cylinder) into the water for 15-min; 24 h later, the rats were returned to the water-filled cylinder and tested for another 5 min. Water was changed after every swim session, so that every rat swam in clean water. Rats were videotaped during both test days and

immobility was scored using the Ethovision ® XT 8.0 software (Noldus, Wageningen, The Netherlands). 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 (Cryan *et al.*, 2005).

Radiotelemetry to assess cardiovascular function: In addition to assessing behavioral effects of manipulating dietary methyl donor content in adult male WKY rats, we also used radiotelemetry to monitor baseline and stress-evoked cardiovascular measures (e.g., heart rate [HR], HR variability [HRV], blood pressure) in WKY-DEP and WKY-SUP rats. We hypothesized that WKY-SUP rats would exhibit cardiovascular changes (relative to WKY-DEP) that resembled physiological alterations observed in MS-180-exposed WKY offspring.

At the conclusion of behavior testing, a subset of WKY males from the SUP and DEP groups (n = 7/condition) were randomly selected 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.

Following the one week surgical recovery, rats were housed in a 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), HR, and locomotor activity were recorded over a 24-h period during week seven to eight of the study. Continuous blood pressure recordings were acquired with a sampling rate of 500 Hz with data averaged over ten seconds. Rats were kept undisturbed during recording sessions. Data were extracted from the DSI ART 4.3 analysis software as 30-min moving averages. HR, BP and activity recordings were analyzed separately during the 12 h light (inactive) and the 12 h dark (active) phases of the 24 h cycle. HRV was analyzed in the time domain and was calculated from the 24 h continuous blood pressure recordings that were binned into 5-min 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-min segments. Data was averaged during the 12-h light phase and 12-h dark phase and further analyzed.

One week after the baseline cardiovascular measures were collected, rats were exposed to homecage intruder stress during the midpoint of the light phase of the 24 h cycle to determine stress-evoked cardiovascular responsivity in WKY-SUP versus WKY-DEP rats. Experimental rats (WKY-SUP or WKY-DEP animals) were subjected to homecage intrusion stress when an age-matched socially-housed intruder rats was introduced into their homecage for ten min. We sampled blood pressure and HR at 500 Hz and digitized data as ten second moving averages. Data were then averaged over one-min bins and extracted for offline analyses. Data were acquired for three epochs: *(1)* an initial baseline period 1-2 h before the intruder was introduced;

(2) ten min during the home cage intrusion; and then *(3)* two h following intruder exposure to assess recovery. Maximal responses for HR and MAP in terms of percent change from baseline were calculated for each animal. We also 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 Y0 is maximal MAP or HR, K is the rate constant/decay constant, and X is time.

Tissue collection and processing: One day after the final cardiovascular assessment, WKY-DEP and WKY-SUP rats were euthanized by rapid decapitation to collect brain tissue, which was removed, flash frozen in isopentane cooled to -30°C on dry ice, and then stored at -80°C. Tissue punches from the hippocampus were collected from each rat as described above. 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.

Statistical analysis

Data analyses for the MethylCap-seq experiment are described above. Data from the global DNA methylation (5-methylcytosine) assessment, qRT-PCR study, and methyl donor diet behavioral and cardiovascular studies were analyzed using GraphPad Prism Software (Version 6.0 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). All data sets were first verified to be normally distributed using the D'Agostino & Pearson omnibus normality test. In the methyl donor diet manipulation studies, rats' weight was analyzed by two-way ANOVA, with age and diet condition as independent variables. Data from the qRT-PCR and behavioral studies were analyzed with a two-tailed Student's t-test, or with the Mann-Whitney U test if not normally distributed. For all analyses, $\alpha<0.05$. Because some of the samples used in

the global DNA methylation assay were derived from animals from the same litter, a covariate analysis was performed using litter as an additional factor. Accordingly, ANCOVA was performed using SPSS (Version 24) with MS/NH treatment as the independent variable, litter as covariate, and 5-methylcytosine as dependent variable. Blood pressure and HR data averaged over 30 min 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.

Results

Early-life MS elicits global DNA hypermethylation in the hippocampus of WKY offspring.

Since early-life experiences are known to shape adult behavior and physiological stress responses, in part, via DNA methylation modifications in brain regions that regulate stress responsivity (Weaver *et al.*, 2002a; Meaney & Szyf, 2005; Szyf *et al.*, 2005; Weaver *et al.*, 2006), we quantified global DNA methylation (5-methylcytosine) levels in several limbic brain regions (hippocampus, PVN of the hypothalamus, amygdala, BNST, septum, and mPFC) in MS-180-exposed vs. control WKY adult males. MS-180-exposure elicited a dramatic increase in DNA methylation specifically in the hippocampus of WKY offspring compared to NH-exposed controls (F=35.257, p< 0.001). Litter was not a significant source of variation (F=1.114, p = 0.31). There was no effect of MS-180 on 5-methylcytosine levels in the PVN ($t_{(8)}$ = 1.59, *n.s.*), mPFC ($t_{(15)}$ = 0.60, *n.s.*), amygdala ($t_{(16)}$ = 0.683, *n.s.*), septum ($t_{(16)}$ = 1.46, *n.s.*), or BNST ($t_{(8)}$ = 0.78, *n.s.*; **Fig. 1**).

Next-generation sequencing reveals disparate genome-wide as well as gene loci-specific DNA methylation patterns in the MS- versus NH-exposed WKY hippocampus.

To delve more deeply into DNA methylation changes elicited in the hippocampus of MS-180-exposed WKY male offspring, we utilized next-generation methylome sequencing. This technology allowed us to sequence methylated DNA fragments with the resolution of ~300 bp. High-throughput sequencing collected >25M reads per sample, which were mapped onto the rat genome reference sequence (rn5), and analyzed with MACS peak calling to examine total number of sites enriched for DNA methylation. This analysis identified 308,904 methylated genomic sites. These sites were subjected to further analysis to identify the sites that displayed differential methylation between MS-180 and control groups. For analysis of these data, we chose to use two methods: 1) first, we subjected the data to stringent thresholds to obtain only the sites with the high level of differential methylation, 2) we utilized more liberal statistical thresholds to allow identification of possible patterns within genetic elements and to find groups of genes that were enriched in differentially DNA methylation in MS/NH samples.

To visualize the occurrence of differentially methylated regions (DMRs) across the genome, we created a Manhattan Plot (**Fig. 2**). We examined genes that surpassed two thresholds: *a)* a threshold p-value < 1×10^{-5}, the suggested threshold in genome-wide studies (indicated by a blue line in **Fig. 2**); and then *b)* a second more stringent threshold p-value < 5×10^{-8} (indicated by a red line in **Fig. 2**). A group of 56 DMRs, which included 15 gene regulatory sites (a region within the gene body or up to 10 kb upstream of a gene) surpassed the lower (blue line) threshold. A group of 40 DMRs within 12 gene regulatory sites passed the the more conservative analytical parameters of the higher (red line) threshold. Some example genes are displayed in the plots in **Fig. 2**, including: *Caln1*, *Map3k5*, *Insr* and *Ephb1*.

We also visualized MS-180/control hippocampal methylation differences by creating a correlational matrix with intensity levels for methylation differences (normalized reads per kb) across 308,904 genomic sites (MS-180 samples represented along the y-axis and NH samples along the x-axis). There was a strong correlation in signal across these sites (*R=0.981*; **Fig. 3A**).

Our next phase of analysis determined the number of MS-180/NH DMRs ($p < 0.1$ BH correction) within specific genomic features (**3B-E**), first focusing on CpG islands, defined as >200 base pair stretches of DNA with GC percentage greater than 50% and observed-to-expected CpG ratio greater than 0.6 (**Fig. 3B-D**). Of the 983 DMRs identified between MS-180 and control hippocampal samples, 7.93% of those sites were within CpG islands; 1.63% of the sites were located within CpG shores (0-2 kb from CpG island edges); and 2.14% of the sites were located within CpG shelves (2-4 kb from island edges). Thus, the majority of DMRs were located in "open sea", defined as genomic regions >4 kb from any CpG island edge (**Fig. 3C**). We also separately considered DMRs within regions located either upstream ("north") or downstream ("south") of CpG islands, considering relative methylation level (normalized reads per kb) in MS-180 versus control samples. Here, we found that, on average, sites within CpG islands as well as north and south shelves were hypomethylated in MS-180 versus control samples, while sites within north and south shores are hypermethylated in MS-180 versus control (**Fig. 3D**). We next annotated methylation data based the type of genomic element where DMRs were located, including within: 10 kb or 2 kb upstream of the transcription start site (TSS), the gene body, 2 kb or 10 kb downstream. On average, sites within regions 2 kb upstream or downstream of a gene body were hypermethylated in MS-180 compared to control samples; DMRs located within gene bodies, 10 kb upstream or 10 kb downstream of genes were hypomethylated. While many studies of MS-180 investigate the differentially methylation at gene promoters, our data show that the highest number of DMRs was located within intra- and inter- genic regions (**Fig. 3E**).

We next performed Gene Ontology (GO) analysis on the 983 DMRs (which corresponded to 46 mapped genes) and identified several biologically relevant ontological terms that were significantly over-represented among the genes differentially methylated in the MS-180-exposed versus control WKY hippocampus. **Table 2** shows a list of the top ten GO terms and associated genes, which are involved in a host of neuronal processes including: cell

proliferation, axonal guidance, tyrosine kinase signaling, and synaptogenesis and synaptic transmission. **Table 3** provides details on the methylation differences detected within these genes. From this gene list we selected four genes – the insulin receptor preproprotein gene (*Insr*), two molecules downstream of insulin receptor signaling (*Map3k5* and *Igf1r*), and glutamate receptor kainate-4 (*Grik4*) – for follow-up qRT-PCR experiments to test whether observed MS-180-induced methylation changes within these genes was accompanied by a significant increase in mRNA levels. Our MethylCap-seq revealed hypomethylated DMRs in *Insr* and *Map3k5* and hypermethylated DMRs in *Igf1r* and *Grik4.* Our results revealed a 1.60 fold increase in hippocampal *Insr* mRNA levels in MS-180-exposed WKY hippocampus relative to control (t_8= 2.87, p< 0.05). For *Map3k5*, we found a 1.40 fold change increase in MS-180 vs. NH rats (t_8=2.53, p<0.05). For *Igf1r*, we found a 1.45 fold change increase in MS-180 vs. NH rats (t_8=3.37, p<0.01). For *Grik4*, we found a 1.49 fold change increase in MS-180 vs. NH rats (t_8=3.98, p<0.01). As negative controls, we also measured the gene expression in *Jmjd8* (t_8= 0.9431, n=5, p = 0.37) and *Dnaz* (t_8= 0.02514, p = 0.98). These results are summarized in **Table 4**.

Increasing methylation (via dietary methyl donor supplementation) improves WKY rats' behavior and physiology, closely resembling the effects of MS.

To begin to examine the functional implications of MS-180-elicited increases in DNA methylation, we manipulated dietary methyl donor content in diet of adult WKY males (by either depleting methyl donor content or supplementing it). Since our prior studies found that MS-180 exposure produced several adaptive behavioral and physiological changes in WKY offspring together with dramatically increased hippocampal DNA methylation, we predicted that dietary methyl donor supplementation would mimic these adaptive effects. At the conclusion of behavioral and cardiovascular assessments in WKY rats receiving either a diet enriched with

(WKY-SUP) or 90% depleted of (WKY-DEP) methyl group donors, brain tissue was collected. To test the efficacy of the diet manipulation, we assessed amino acid content within the hippocampus of WKY-SUP and WKY-DEP rats and found that methionine levels were increased by 31% in WKY-SUP versus WKY-DEP brain tissue (WKY-SUP 2.48 ± 0.11 versus WKY-DEP 1.89± 0.11; t_{14}=3.74, p=0.002).

Effect of methyl donor diet manipulation on body weight. As noted in the Methods section, all groups were food-restricted to ensure equal feeding between groups since previous studies found significantly diminished food intake in rodents receiving a methyl-depleted diet (Konycheva *et al.*, 2011; Ishii *et al.*, 2014). The WKY-SUP and WKY-DEP groups were weighed once per week starting on the first day of diet exposure through the end of behavior testing. All rats gained weight during the diet manipulation period (effect of time [$F_{12, 312}$=1211, p<0.0001]), although WKY-SUP rats were consistently heavier than WKY-DEP rats (WKY-SUP average weight over the course of the study was 219±2 g versus WKY-DEP average weight of 185±2 g; effect of diet [$F_{1, 26}$=119, p<0.0001]), which is consistent with previous reports (Konycheva *et al.*, 2011; Ishii *et al.*, 2014).

Behavioral effects of increasing methylation via dietary methyl donor supplementation. Adult WKY males that were subjected to either the methyl donor supplemented or depleted diets were evaluated in multiple tests of anxiety- and depression-like behavior. WKY rats that received the methyl donor supplemented diet (WKY-SUP) showed greater exploratory behavior in the open field (OF), traversing a greater distance during the test ($t_{(26)}$ = 1.89, p<0.05), showing more rearing ($t_{(26)}$ = 1.93, p<0.05), and decreased grooming behavior ($t_{(26)}$ = 1.87, p<0.05) compared to WKY rats receiving a methyl donor depleted diet (WKY-DEP). Although both groups spent a similar amount of time in the center of the OF ($t_{(26)}$ = 0.71, p=0.24; **Fig. 4A**). In the social interaction test, WKY-SUP rats made more frequent visits to a male social stimulus rat

compared to WKY-DEP rats ($t_{(26)} = 2.33$, $p<0.05$); both groups made a similar number of visits to an inanimate novel object ($t_{(26)} = 0.57$, n.s.; **Fig. 4B**). In the FST, WKY-SUP rats showed less depression-like behavior compared to WKY-DEP rats, spending significantly less time immobile ($t_{(26)} = 3.65$, $p<0.001$) and greater swimming velocity ($t_{(26)} = 4.76$, $p<0.0001$; **Fig. 4C**).

Baseline cardiovascular effects of increasing methylation via dietary methyl donor supplementation. For resting HR, WKY rats that received a methyl-donor supplemented diet (WKY-SUP) showed decreased HR during both the light ($t_{(12)} = 8.96$, $p<0.0001$) and dark phase ($t_{(12)} = 8.63$, $p<0.0001$) of the 24 h cycle compared to methyl donor depleted rats (WKY-DEP; **Fig. 5A**). These diet-induced decreases in resting HR were accompanied increased HRV (SDNN) in WKY-SUP versus WKY-DEP rats during the light phase ($t_{(12)} = 2.63$, $p<0.05$), but not the dark phase ($t_{(12)} = 0.69$, *n.s.*; **Fig. 5B**). Dietary methyl donor supplementation also decreased resting systolic blood pressure (SBP) and resting diastolic blood pressure (DBP) relative to WKY-DEP rats during both the light phase (SBP: $t_{(12)} = 3.94$, $p<0.001$; DBP: $t_{(12)} = 2.27$, $p<0.05$) and dark phase (SBP: $t_{(12)} = 4.91$, $p<0.001$; DBP: $t_{(12)} = 3.71$, $p<0.01$) of the 24 h cycle (**Fig. 5C**-**D**). **Supplemental Fig. 1** presents HR and BP data in 30-min increments for SUP and DEP rats across the 24-h assessment period.

Stress-Evoked Cardiovascular Responses. Rats were briefly exposed to 10 min home cage intrusion when a novel age-matched male intruder rat entered their 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. This analysis revealed significantly different curves that best represent HR recovery data between groups ($F_{3, 1688} = 14.28$, $p < 0.0001$). The R^2 (i.e. goodness of fit index) for each curve was 0.33 and 0.49 for DEP and SUP groups, respectively, with the following estimated values: Y0 – 45.05 (DEP), 70.86 (SUP); Plateau – -1.94 (DEP),

0.88 (SUP), K – 0.045 (DEP), 0.076 (SUP). Data from individual rats were then curve fitted to estimate group differences in specific parameters of the recovery. Although SUP and DEP groups displayed similar maximal HR responses following home cage intrusion, SUP rats exhibited greater decay constant than the WKY-DEP group ($t_{(12)} = 4.37$, $p < 0.001$), indicating faster HR recovery (**Fig. 6A**). Mean Arterial Pressure (MAP) responses to intruder exposure were also assessed in a similar fashion to HR data. Curve-fitting of grouped data ($R^2 = 0.45$ DEP, $R^2 = 0.45$ SUP) showed a significant difference between groups ($F_{3,1688} = 4.268$, $p = 0.0052$) with the following estimated values: Y0 – 36.72 (DEP), 38.19 (SUP); Plateau – -2.49 (DEP), -0.31 (SUP), K – 0.055 (DEP), 0.073 (SUP). Although SUP and DEP groups showed similar maximal MAP responses to home cage intrusion, SUP rats exhibited a modest, albeit significant increase in the decay constant ($t_{(12)} = 2.45$, $p < 0.05$), suggesting faster MAP recovery (**Fig. 6B**).

Discussion

Stressful experiences during early life can shape the developing brain and lead to life-long behavioral and physiological effects (Heim *et al.*, 2002; Apter-Levy *et al.*, 2013). Although a preponderance of studies focuses on the harmful effects of early-life stress, accumulating evidence suggests that such experiences can lead to positive effects in certain individuals (Gluckman *et al.*, 2007; Santarelli *et al.*, 2014; Rana *et al.*, 2015). WKY rats are known to be a highly stress-susceptible strain, exhibiting abnormalities within behavioral domains commonly associated with depression and anxiety disorders, high levels of hypothalamic pituitary adrenal axis (HPA) stress reactivity, as well as proclivity to stress-induced ulcers (Pare & Redei, 1993; Malkesman *et al.*, 2006; Overstreet, 2012; Nam *et al.*, 2014). Although WKY rats are vulnerable to stress exposure during adulthood, our recent study showed that early-life MS-180 elicits protective effects in WKYs, leading to increased exploratory behavior, enhanced sociability,

decreased anxiety-like behavior, diminished helplessness in the FST (Rana *et al.*, 2015), while producing the opposite effects in the stress resilient Wistar rats (Rana *et al.*, 2015). These positive behavioral changes are accompanied by adaptive cardiovascular alterations (i.e. decreased baseline HR and increased HRV) (Rana *et al.*, 2016). The present experiments aimed to identify neural and molecular mechanisms that may confer these adaptive behavioral and physiological changes in MS-180-exposed WKY offspring. We found that MS-180 elicits a dramatic increase in global DNA methylation in the hippocampus, but not other brain regions studied, suggesting that this epigenetic process contributes to the effects of MS-180 in WKY rats. Next-generation sequencing identified a number of gene-specific methylation differences in the MS-180-exposed WKY hippocampus, including altered methylation of genes encoding the insulin receptor and its downstream targets. Our final experiment manipulated DNA methylation in WKY rats by depleting or supplementing dietary methyl donor content to test the hypothesis that enhancing DNA methylation in brains of adult WKY rats (via increased dietary methyl donor content) would recapitulate behavioral and cardiovascular changes akin to the effects of MS-180. As predicted, dietary methyl donor supplementation improved WKY's anxiety- and depression-like behavior and improved cardiovascular parameters relative to dietary methyl donor-depleted WKY rats. Overall these data suggest a potential molecular mechanism that mediates a PAR whereby early-life stress induces DNA methylation changes in the brain that may contribute to successful stress coping in adulthood.

MS-exposure specifically affected WKY hippocampus, with no significant methylation changes in other limbic brain regions.

Early-life MS-180 has been shown to elicit a number of structural and molecular changes in the developing and adult brain, particularly in the hippocampus, prefrontal cortex, and hypothalamus. For example, MS-180 was found to alter hippocampal neurogenesis (Lajud *et al.*, 2012), cell proliferation (Hulshof *et al.*, 2011), dendritic length (Monroy *et al.*, 2010), dendritic

spine density (Monroy *et al.*, 2010), and mossy fiber density within the hippocampal subfields (Huot *et al.*, 2002). These structural changes appear to be accompanied by a host of molecular alterations, including altered expression of glucocorticoid receptors (Meaney *et al.*, 1996; Ladd *et al.*, 2004), glutamate receptor subunits (Pickering *et al.*, 2006), histone deacetylases (Suri *et al.*, 2014), and histone methyltransferases (Suri *et al.*, 2014) along with increased histone methylation (Suri *et al.*, 2013) and hypermethylation of the glucocorticoid receptor gene (Kundakovic *et al.*, 2013). MS-180 exposure has also been shown to trigger structural and molecular changes in the prefrontal cortex, including decreased dendritic length and spine density (Monroy *et al.*, 2010), atrophy of basal dendritic tree and reduced spine density apical/basal dendrites in layer II/III pyramidal neurons, impaired long-term potentiation, and increased expression of AMPA glutamate receptor subunits (GLUR1 and GLUR2), PSD95 and CamKII (Chocyk *et al.*, 2013). In the hypothalamus, aside from MS-180-induced changes in corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP) production (Plotsky & Meaney, 1993; Ladd *et al.*, 2005; Plotsky *et al.*, 2005), early-life MS-180 also leads to greater overall volume of the PVN and supraoptic nucleus as well as decreased serotonergic innervation of the hypothalamus (Lukas *et al.*, 2010). Based on these previous findings of MS-180 impacting multiple limbic regions, our first experiment examined possible MS-180-induced global DNA methylation differences in several brain areas (the hippocampus, mPFC, PVN of the hypothalamus as well as the amygdala, septum, and BNST) of MS-180-exposed and control WKY adult males. MS-180 exposure triggered a robust increase in global methylation selectively in the hippocampus of WKY rats, with no significant changes occurring in the other areas.

Our finding of increased DNA methylation levels specifically in the hippocampus of adult MS-180-exposed WKYs suggests that the adaptive behavioral and cardiovascular effects of MS-180 within WKY offspring may be mediated by changes in hippocampal function. Extensive evidence implicates a role for the hippocampus in regulating each of the behavioral domains

that were affected in MS-180-exposed WKY rats, including exploratory behavior, sociability, and FST immobility (Kimble, 1968; Altman *et al.*, 1973; Wallace *et al.*, 1977; Sherman & Petty, 1980; Sams-Dodd *et al.*, 1997; Becker *et al.*, 1999; Joca *et al.*, 2003; Hajszan *et al.*, 2009; Shishkina *et al.*, 2010; Telensky *et al.*, 2011). These behaviors are mediated by multiple hippocampal outflow circuits, including a projection that reaches PVN via a synaptic relay within BNST (Herman *et al.*, 1989; Herman *et al.*, 1996; Herman & Mueller, 2006). Because the PVN also contributes to regulation of cardiovascular function via neurons with direct projections to autonomic regulatory regions (Porter & Brody, 1985; Kerman, 2008; Michelini & Stern, 2009), the cardiovascular effects of MS-180 in WKY rats may be mediated via a hippocampal projection to PVN.

Impact of MS on DNA methylation in brain

Environmental factors and various life experiences modify DNA methylation and other 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; Klengel *et al.*, 2014). Most studies to-date that examine neural DNA methylation changes following early-life adversity focus on genes related to the HPA stress axis, such as the glucocorticoid receptor (GR), CRH, and AVP (Murgatroyd, 2014; Bockmuhl *et al.*, 2015; Palma-Gudiel *et al.*, 2015; van der Doelen *et al.*, 2015; Turecki & Meaney, 2016). Fewer studies have reported methylation changes on a genome-wide scale. Work in humans has largely been restricted to examining genome-wide methylation patterns in peripheral samples (i.e. whole blood, buccal cells) collected from children exposed to stress or abuse (Klengel *et al.*, 2014; Lutz & Turecki, 2014). Rodent studies have begun to evaluate global methylation changes in the brains of rats or mice exposed to early-life stress, although results vary across studies based on the specific stress paradigm used, strain, sex, or age of offspring studied (Kember *et al.*, 2012; Anier *et al.*, 2014; Doherty *et al.*, 2016). For example, one study

found that exposing Wistar rats to MS-180 in early life led to decreased global DNA methylation levels in the nucleus accumbens of adult offspring (Anier *et al.*, 2014). Another study utilized a different early-life adversity model (transiently exposing Long-Evans rat pups to an abusive caregiver) and showed that it increased global DNA methylation levels in the hippocampus of adolescent male offspring (Doherty *et al.*, 2016). The current study revealed a robust increase in global DNA methylation in the hippocampus of adult WKY male offspring that were exposed to daily 3-h MS from P1-14. Our next-generation sequencing results corroborated this MS-180-induced increase in methylation and identified 983 genomic regions that were differentially methylated in the MS-180 versus control WKY hippocampus. The majority of DMRs occurred within exons or intergenic regions, with approximately 10% of the DMRs localized in or around CpG islands. Our analysis identified a number of differentially methylated genes, including molecules involved in cell proliferation, axonal guidance, tyrosine kinase signaling, and synaptogenesis and synaptic transmission.

Although our analysis identified numerous genes that were differentially methylated in the hippocampus of MS-exposed offspring compared to controls, it was interesting to find that certain genes previously shown to be sensitive to early life stress and maternal care (such as the glucocorticoid receptor gene, Nr3c1) were not affected in our study. Other groups found that Nr3c1 methylation is regulated by naturally-occurring differences in maternal care (Weaver *et al.*, 2002b), although there are conflicting reports of the effect of early life stress on Nr3c1 methylation. Two such studies reported MS-induced increase in NR2c1 methylation in DBA/6J and C57BL/6J males (Kember *et al.*, 2012; Kundakovic *et al.*, 2013), but Kember et al. found no change in C57BL/6J mice and Kundakovic found no differences in Balb/cJ mice. These disparate results (as well as the lack of NR2c1 methylation changes in our present experiment) are likely related to different mouse or rat strains used across studies as well as technical differences in the maternal separation protocols strain (Kember *et al.*, 2012; Kundakovic *et al.*, 2013).

One of the top molecular pathways that emerged from our methylome analysis of the MS-180-exposed WKY hippocampus involved genes related to insulin receptor signaling. Our analysis revealed large and significant ($p < 10^{-6}$) MS-180-induced methylation decreases at intragenic sites within two key nodes of insulin signaling pathway: *a)* the insulin receptor (Insr) and *b)* mitogen activated protein kinase kinase kinase 5 (Map3k5), a major downstream target of Insr. It may be surprising that we found MS-associated hypomethylation at sites related to these genes when the global methylation assay (data presented in **Fig. 1**) pointed to dramatic MS-induced hypermethylation. Methylation patterns can vary dramatically across the genome and environmental factors such as MS exposure likely elicit disparate effects across the genome, with the potential for increasing methylation at some points, but decreasing it elsewhere. There are host of molecular processes and structural aspects of certain genomic regions that regulate methylation, which may provide protection of select genes (such as insulin-related genes) against MS-related hypermethylation. For example, the presence and functionality of distinct DNA methyltransferase enzymes, transcription factors, and methyl binding proteins at distinct gene sites may lead to differential regulation of methylation status and later transcription. To the best of our knowledge, these factors are not well understood for insulin-related genes identified in our analyses, although we are certainly interested to pursue such topics in our ongoing work related to this project.

Our qRT-PCR experiment indicated that hypomethylation of sites within the insulin receptor gene was associated with altered gene expression, with a 1.62-fold increase in *Insr* mRNA levels in the hippocampus of MS-180 versus control groups. Insulin signaling in the brain has been implicated in diverse functions, including metabolism, reproduction, memory, and neuronal survival (Plum *et al.*, 2005). Brain-specific deletion of insulin receptor precipitates depressive- and anxiety- like behaviors in mice, which are reversible with antidepressant drug treatment (Kleinridders *et al.*, 2015). Insulin signaling has also been implicated in hippocampal function, with insulin administration in humans leading to improved hippocampal-dependent

verbal memory and delayed word recall (Benedict *et al.*, 2004; Reger *et al.*, 2006; Benedict *et al.*, 2007; Hallschmid *et al.*, 2008; Reger *et al.*, 2008) as well as improved mood (Benedict *et al.*, 2004; Hallschmid *et al.*, 2008). Results from rodent studies are consistent with these findings, showing that injecting insulin into the brain improves performance on hippocampal-dependent tasks, including memory consolidation and spatial memory (Park *et al.*, 2000; Moosavi *et al.*, 2006; Moosavi *et al.*, 2007; Haj-ali *et al.*, 2009; McNay *et al.*, 2010), and memory in a passive-avoidance task (Babri *et al.*, 2007). Future experiments in our laboratory plan to explore the role of insulin receptor signaling in mediating the adaptive effects of MS-180 on WKY behavior.

Increasing dietary methyl donor content leads to improved anxiety- and depressive-like behavior as well as positive cardiovascular effects in WKY rats

Diet represents one of several environmental factors that can potently influence DNA methylation since dietary folate, choline, and methionine act as methyl donors for one-carbon transfer reactions like DNA methylation. DNA methyltransferases (DNMTs) transfer methyl groups from S-adenosylmethionine (SAM) to cytosine (Jones & Takai, 2001), so diets lacking folate or other methyl donors can impede SAM synthesis, thereby leading to DNA hypomethylation (Ghoshal *et al.*, 2006; Pogribny *et al.*, 2008; Chen *et al.*, 2010). Depleting dietary methyl donor content has been shown to decrease DNA methylation markers in the brain (Pogribny *et al.*, 2008), and impede fear memory (Ishii *et al.*, 2014; Tomizawa *et al.*, 2015). On the other hand, boosting levels of methyl donors (or SAM itself) increases DNA methylation levels in brain (Paternain *et al.*, 2016), and elicits antidepressant effects in rats and mice, such as decreased immobility in the FST and improved stress-induced anhedonia (Czyrak *et al.*, 1992; Benelli *et al.*, 1999; Brocardo *et al.*, 2008; Molina-Hernandez *et al.*, 2011; Paternain *et al.*, 2016). The present study showed that increasing WKY male rats' dietary methyl donor content elicited a series of positive behavioral effects including: increased exploratory behavior, increased social interaction, and decreased behavioral despair (immobility) in the FST. These

data together with results from other rodent studies are generally consistent with findings in humans showing that treatment with DNA methylation-promoting agents (i.e. SAM (Delle Chiaie *et al.*, 2002; Mischoulon & Fava, 2002; Hardy *et al.*, 2003) or L-methylfolate (Papakostas *et al.*, 2012; Roberts & Tranter, 2013; Shelton *et al.*, 2013)) improves depressive symptoms in some patients.

Increased methyl donor content also leads to multiple cardiovascular changes in WKY rats, including decreased resting HR, increased HRV, decreased resting blood, as well as improved cardiovascular recovery to social stress (increased decay constants for HR and MAP following intruder exposure). These findings are similar to a study in Wistar-Hanover rats where a methionine-enriched diet led to decreased MAP (Mariotti *et al.*, 2006), although other studies have reported that a methionine-enriched diet increases SBP in other rat strains (Robin *et al.*, 2003; Robin *et al.*, 2004). Taken together these observations suggest that the effects of methyl donor supplementation in rats may depend on the strain and on baseline blood pressure levels. Interestingly, in clinical populations folate supplementation leads to a decrease in stroke incidence in hypertensive individuals (Huo *et al.*, 2015). It is thought that these effects are mediated by changes in circulating homocysteine levels and their effects of vascular function and plaque formation (Santilli *et al.*, 2016). Our present results raise the possibility that such protective effects may be mediated by DNA methylation changes in the brain. Future studies in which we alter methyl donor availability only in the brain will be required to address this issue. Additional studies should also determine whether the methyl donor supplemented diet changes hippocampal gene expression within genes that were found to be altered by MS-180 exposure; because MS-180 exposure and the diet manipulation induce similar behavioral and cardiovascular effects, it would be interesting to determine whether they potentially act through similar molecular mechanisms in the hippocampus.

Technical considerations

An important limitation of the present study is the fact that our experiments used only male WKY offspring. There is a relative lack of information about epigenetic differences in the developing and adult brains of males versus females (Menger *et al.*, 2010; McCarthy & Nugent, 2015) and how such differences may contribute to sexually dimorphic risk for emotional disorders (Uddin *et al.*, 2013). Thus, it will be important for studies like the current one to determine whether early-life stress elicits distinct epigenetic changes in female versus male offspring and how those differences may impact behavioral outcomes. Future experiments in our laboratory aim to determine whether MS-180 elicits similar neural, behavioral and physiological effects in WKY females, and whether methyl donor depletion and/or supplementation influences WKY females as it does in males.

The repertoire of techniques for interrogating genome-wide patterns of epigenomic marks is rapidly developing. Each technique has strengths and weaknesses, and each offers distinct types of information that contribute to our understanding of epigenome structure and function (Harris *et al.*, 2010). We chose MethylCap-Seq because it offers a balance between cost and genome coverage. A limitation of MethylCap-Seq is that it does not offer single-base resolution and shows preference for CpG dinucleotides, whereas bisulfite-based methods can offer this resolution without the preference. On the other hand, it is noteworthy that an advantage of MethylCap-Seq over bisulfite sequencing is that it offers a clean 5-methylcytosine signal whereas bisulfite sequencing cannot resolve 5-methylcytosine and 5-hydroxymethylcytosine without further oxidative steps. Another caveat of our approach is that our tissue punch samples contain a heterogeneous collection of cells (i.e. neurons and glia) that exhibit distinct epigenetic patterns (Lister *et al.*, 2013). Because MethylCap-Seq requires a large amount of genomic DNA, tissue punches are preferable to methods such as cell-sorting techniques or laser-capture microdissection of distinct cell populations. As the techniques improve, we hope to be able to sequence from these specific cell populations. A future goal will be to identify how epigenetic

and transcriptional sites differ within cell populations in these brain regions.

One limitation of the current study is that we attempted to manipulate brain DNA methylation by exposing rats to a diet that is either supplemented with or depleted of methyl group donors. Our observation of the significantly greater methionine levels in the hippocampi of the methyl donor supplemented rats suggests increased DNA methylation in the brains of these animals (Anderson *et al.*, 2012). However, it is also likely that this diet-based manipulation also impacted DNA methylation levels throughout the body in a variety of tissues. Two prior studies reported that prenatal exposure to a methyl donor supplemented diet increased the occurrence of colitis coupled with microbiome and epigenetic changes in colonic mucosal tissue of adult offspring (Schaible *et al.*, 2011; Mir *et al.*, 2013). However, these studies did not assess behavioral outcomes, and we are not aware of such studies using the adult diet manipulation akin to our present experiment. It is also feasible that such effects may not have been restricted to the DNA. For example, availability of methyl groups has been suggested to alter circulating catecholamine levels, because methyl group availability may impact the function of methyltransferases that regulate catecholamine synthesis and breakdown (Zhou *et al.*, 2011). Future studies that utilize manipulations to specifically alter DNA methylation levels in the brain will help address this issue.

Conclusions

In summary, our recent work found that exposing stress-reactive WKY rats to early-life MS-180 stress elicited paradoxically positive behavioral and physiological effects, including reduced levels of anxiety- and depressive-like behavior (Rana *et al.*, 2015). Results of the present studies suggest that the adaptive effects of MS-180 in WKY offspring may be mediated, at least in part, through MS-180-induced DNA methylation changes in the hippocampus. MS-180 exposure led to significantly elevated total DNA methylation specifically in the hippocampus of WKY male offspring; moreover, enhancing DNA methylation levels in adult WKY rats (via

dietary methyl donor supplementation), elicited positive behavioral and cardiovascular effects that resembled the effects of MS-180. Next-generation sequencing analysis identified a number of molecular pathways with genes that were differentially methylated following MS-180 exposure, including molecules important in insulin receptor signaling. Future experiments will continue to interrogate molecular processes, including DNA methylation as well as insulin receptor signaling, that may contribute to the adaptive effects of MS-180 in WKY offspring and increased stress resilience.

Acknowledgements

The authors would like to thank Nateka Jackson for excellent technical assistance with the behavioral studies. The authors would like to thank Dr. David Pollock and Dr. Jennifer Pollock for their help with radiotelemetry studies. The study was funded by: NIMH R00 MH081927-04 (IAK); American Heart Association Predoctoral Fellowship 13PRE16940050 (SR); NIH 4R00MH085859-02 (SMC), NIH R01MH105447-01 (SMC), P30 NS047466PI (JMW), and 5T90DE022736-04 (covering CRM). The authors declare no competing financial interests.

Figure Legends

Figure 1. Effects of neonatal maternal separation (MS) on global DNA methylation (5-methylcytosine) in the adult Wistar Kyoto (WKY) rat brain. WKY rats were exposed to daily 180-min MS or 15-min neonatal handling (NH) between postnatal days (P) 1-14. Adult offspring were sacrificed and several brain regions were dissected: the hippocampus, paraventricular nucleus (PVN) of the hypothalamus, amygdala, septum, medial prefrontal cortex (mPFC), and bed nucleus of the stria terminalis (BNST). DNA was extracted and used to measure 5-methylcytosine, an indicator of global DNA methylation. MS exposure dramatically increased 5-methylcytosine levels in the adult WKY hippocampus relative to NH controls, but did not significantly alter DNA methylation levels in the PVN, mPFC, amygdala, septum or BNST Data represent mean ± SEM. **** Statistically significant differences at $p < 0.0001$.

Figure 2. Manhattan plot illustrating differentially methylated sites in the adult hippocampus of MS-exposed versus control WKY rats. Differentially methylated regions (DMRs) are shown by chromosome (along the x-axis) and the negative logarithm of the association p-value for each block (-log10 [p-value]) on the y-axis). We examined whether DMRs surpassed two thresholds: *a)* a threshold p-value less than 1×10^{-5}, the suggested threshold in genome-wide studies, indicated by a blue line; and then *b)* a second more stringent threshold p-value less than 5×10^{-8}, indicated by a red line. There were 56 DMRs in the MS versus control adult hippocampus that surpassed the lower (blue line) threshold. This group of DMRs included 15 gene regulatory sites (a region within the gene body or up to 10 kb upstream of a gene). When considering the more stringent parameters of the higher (red line) threshold, we found 40 DMRs within 12 gene regulatory sites that passed that threshold. Some example genes that met these criteria are labelled: ephrin type-B receptor 1 (*Ephb1*), insulin receptor preprotein (*Insr*), mitogen-activated protein kinase kinase kinase 5 (*Map3k5*), calcium-binding protein 8 (*Caln1*)

Figure 3. Early-life maternal separation (MS) exposure elicits lasting DNA methylation changes within several genomic regions of WKY hippocampus. **(A)** Correlation analysis demonstrated a strong correlation in signal across 308,904 genomic sites that were differentially between samples from MS-exposed and control rats. MS samples represented along the y-axis and neonatal handle control samples along the x-axis. The shift towards increased signal intensity above the diagonal line indicates greater methylation signal in MS versus control hippocampal samples. **(B)** Graphic depicts an example CpG Island (in red) surrounded by CpG island north and south shores (in green, 0-2 kb from CpG island edges) and CpG island north and south shelves (in purple, 2-4 kb from island edges). Genomic regions >4 kb from any CpG island edge is considered "open sea". **(C)** Of the 983 differentially methylated regions (DMRs) between MS and control WKY hippocampal samples, the majority were located in "open sea", with approximately 12% of the DMRs occurring within CpG islands, shores and shelves. **(D)** Within the CpG islands and surrounding shores and shelves, sites within the CpG islands, north and south shelves were hypomethylated in MS versus control samples, while sites within north and south shores were hypermethylated between the groups. **(E)** Methylation data were also classified depending upon where DMRs fell within specific genomic regions, including within: 10 kb or 2 kb upstream of the transcription start site (TSS), the gene body, 2 kb or 10 kb downstream of the gene body. The highest number of DMRs occurred within exons or intergenic regions. On average, sites within regions 2 kb upstream or downstream of a gene body were hypermethylated in MS compared to control samples; DMRs within gene bodies, 10 kb upstream or 10 kb downstream of genes were hypomethylated.

Figure 4. Dietary methyl donor supplementation in WKY rats has anxiolytic and antidepressant effects. **(A)** WKY rats were fed either a diet depleted (WKY-DEP) of methyl donors (e.g., folate, choline, etc.) or a diet supplemented (WKY-SUP) with excess methyl donors for 4 weeks before behavioral testing commenced and throughout the remainder of the study. In the Open Field

Test, WKY-SUP rats exhibited increased exploration in the novel environment compared to WKY-DEP rats. WKY-SUP rats traversed a greater distance in the open field and spent more time rearing compared to WKY-DEP rats. Although the two groups spent a similar amount of time in the center of the open field, WKY-SUP rats showed less grooming behavior, suggesting a subtle decrease in anxiety-like behavior. **(B)** In the social interaction test, methyl donor supplementation subtly improved WKYs' typically low levels of social interaction. WKY-SUP animals made more frequent visits to the male social stimulus rats compared to the WKY-DEP group, but showed similar exploration of a novel object. **(C)** In the Forced Swim Test (FST), dietary methyl donor supplementation had an antidepressant effect, with WKY-SUP rats showing decreased immobility and greater swimming velocity relative to WKY-DEP rats. Data represent mean ± SEM. *Statistically significant differences at $p < 0.05$; ** indicates $p < 0.001$; *** indicates $p < 0.0001$.

Figure 5. Dietary methyl donor supplementation leads to reduced baseline heart rate (HR), blood pressure (BP), and heart rate variability in WKY rats. **(A)** WKY rats that received a methyl donor-supplemented diet (WKY-SUP) showed substantially lower resting HR compared to those receiving a methyl donor depleted diet (WKY-DEP) during both the light and dark phases of a 24 h period. **(B)** These diet-induced changes in resting HR were accompanied by increased heart rate variability (standard deviation of inter-beat-interval ; SDNN) in WKY-SUP versus WKY-DEP rats during the light phase, but not the dark phase **(C-D)** WKY-SUP rats also showed lower SBP and DBP compared to WKY-DEP rats during the light and dark phases. Data represent mean ± SEM. *Statistically significant differences at $p < 0.05$; ** indicates $p < 0.001$; *** indicates $p < 0.0001$.

Figure 6. Dietary methyl donor supplementation decreases WKY rats' cardiovascular response to acute stress of home cage intrusion. **(A)** A novel age-matched male intruder rat was

introduced into the home cage of each methyl donor supplemented (WKY-SUP) or depleted (WKY-DEP) WKY test rat for 10 min. Maximal heart rate (HR) response was determined by the maximal change in the value from baseline (15 min average prior to the beginning of the test) during the test period. Curve-fitting analysis was also performed to determine HR recovery after stress; solid line indicates curve-fitting analysis in a representative animal. WKY-SUP and WKY-DEP groups displayed similar maximal HR responses to the intrusion, although WKY-SUP rats showed greater HR decay constant, indicating steeper rate of recovery. **(B)** Similarly, the two groups displayed similar maximal mean arterial pressure (MAP) response to home cage intrusion, with the WKY-SUP rats showing increased MAP decay. *Statistically significant differences at $p < 0.05$; ** indicates $p < 0.001$.

Table 1. Composition of methyl-donor manipulated diets.

Dietary component	Methyl-donor supplemented diet (SUP)	Methyl-donor depleted diet (DEP)
Methionine (g)	7.50	0.60
Choline (g)	15.00	0.08
Folic Acid (mg)	15.00	0.20
Zinc (mg)	150.51	29.12
Vitamin B12 (mg)	0.01	0.01
Betaine (g)	15.00	0.00
Methionine (g)	7.50	0.60
Choline (g)	15.00	0.08
Folic Acid (mg)	15.00	0.20
Zinc (mg)	150.51	29.12

WKY rats were randomly assigned to either (1) a methyl donor supplemented (SUP) group receiving a diet enriched with higher than usual amounts of methyl donors and cofactors (folic acid, choline, methionine, and Vitamin B12; diet. No. A04062403, Research Diets Inc, New Brunkswick, NJ; termed methyl donor supplemented group (SUP); or (2) a methyl donor depleted (DEP) group fed chow that was 90% depleted of methyl donors (diet no. A04062402, Research Diets Inc, New Brunkswick, NJ).

Table 2. Biological pathways enriched in genes showing differential methylation in maternal separation versus control WKY rat hippocampal tissue.

Biological Process Pathways		p-value	enrichment ratio	Genes in Pathway
1	positive regulation of cell proliferation	0.008	5.42	Sphk2, Nf1, Nacc1, Cdh13, Peli1, Efnb1, Insr, St8sia1, Ntn1
2	cell proliferation	0.015	3.42	Sphk2, Nf1, Nacc1, Cdh13, Peli1, Efnb1, Insr, St8sia1, Ntn1, Ephb1, Vpreb1, Fgfrl1
3	transmembrane receptor protein tyrosine kinase signaling pathway	0.023	6.62	Cdh13, Efnb1, Insr, Ephb1, Fgfrl1, Atxn1
4	regulation of cell proliferation	0.023	3.53	Sphk2, Nf1, Nacc1, Cdh13, Peli1, Efnb1, Insr, St8sia1, Ntn1, Fgfrl1
5	protein modification process	0.023	3.53	Eya2, Ephb1, Nf1, Peli1, Hus1, Insr, Lrp8, St8sia1, Fgfrl1, Prdm5, Hs3st5, Hecw2
6	macromolecule modification	0.090	2.28	Eya2, Ephb1, Nf1, Peli1, Hus1, Insr, Lrp8, St8sia1, Fgfrl1, Prdm5, Hs3st5, Hecw2
7	locomotion	0.090	2.20	Ephb1, Nf1,Cdh13, Efnb1, Insr, Lrp8, Hs3st5, Ntn1
8	cellular protein modification process	0.090	3.19	Eya2, Ephb1, Nf1, Peli1, Hus1, Insr, Lrp8, St8sia1, Fgfrl1, Prdm5, Hs3st5, Hecw2
9	synaptic transmission	0.090	2.28	Grik4, Ephb1, Nf1, Atxn1, Lrp8
10	regulation of locomotion	0.090	3.98	Nf1, Hs3st5, Cdh13, Insr, Ntn1

This list represents the top ten gene onology (GO) biological processes ($p < 0.1$) along with their associated p-value for enrichment, ratio of enrichment (calculated based on number of genes observed versus those expected), and the genes that appeared within each pathway. The gene list used for this analysis included those containing differentially methylated regions (DMRs) that occurred within the gene body or within 2 kb upstream of gene ($p < 0.1$).

Table 3. Methylation differences in genes enriched in Gene Ontology biological processes

Gene Symbol	Description	Methylation Difference MS-NH (corr reads per million)	Corr p-value	Distance from TSS (bp)
Ephb1	Ephrin type-B receptor 1	-18.918	< 1E-10	73,848
Insr	insulin receptor preproprotein	-13.984	< 1E-10	145,195
Peli1	pellino homolog 1	-0.731	0.00533	160
Fgfrl1	Fibroblast growth factor receptor-like 1	-0.844	0.02583	3,050
Nf1	neurofibromin	0.587	0.04151	115,808
Efnb1	ephrin-B1 precursor	0.643	0.04255	8,937
St8sia1	alpha-N-acetylneuraminide alpha-2,8-sialyltransferase	0.660	0.05003	44,729
Ntn1	netrin-1 precursor	0.658	0.05732	55,487
Nacc1	Nucleus accumbens-associated protein 1	-0.733	0.07153	166
Vpreb1	pre-B lymphocyte 1 precursor	0.588	0.07535	39,103
Sphk2	sphingosine kinase 2	-0.808	0.07799	2,666
Hs3st5	heparan sulfate glucosamine 3-O-sulfotransferase 5	-7.537	0.00029	182,073
Grik4	glutamate receptor, ionotropic kainate 4 precursor	0.613	0.09167	250,769
Atxn1	Ataxin-1	0.622	0.07918	265,815
Lrp8	LDL Receptor Related Protein 8	-0.887	0.00661	41,604
Eya2	eyes absent homolog 2	0.629	0.09848	35,749
Hus1	HUS1 checkpoint Clamp component	0.636	0.05732	4,889
Prdm5	PR domain 5	0.854	0.04823	113,670
Hecw2	E3 ubiquitin-protein ligase	-0.713	0.09527	219,332
Cdh13	cadherin-13 precursor	0.612	0.09065	506,167

The genes listed here were found to be differentially methylated in MS versus NH hippocampus after statistic threshold of $p < 0.1$ and were a part of the top ten GO biological processes shown in Table 3. For each gene, the methylation difference between MS vs. NH groups are displayed in corrected reads per million. For the differentially methylated region (DMR) within the gene, the corrected p-value and the distance of the DMR from the transcription start site (TSS) are listed.

Table 4. Genes showing differential methylation and expression in adult WKY rat hippocampus following maternal separation

Gene Symbol	Description	Difference in methylation MS-NH	Corr P-value	Distance from TSS (kb)	qPCR FC	qPCR p-value
Ephb1	Ephrin type-B receptor 1	-18.918	< 1E-10	73.85	1.05	NS
Insr	insulin receptor preproprotein	-13.984	< 1E-10	145.20	1.60	p < 0.05
Map3k5	mitogen-activated protein kinase kinase kinase 5	-9.045	6.60E-07	146.41	1.40	p < 0.05
Caln1	Calcium-binding protein 8	0.994	2.73E-06	336.07		
Grik4	glutamate receptor, ionotropic kainate 4 precursor	0.613	0.092	250.77	1.49	p < 0.01
Igf1r	Insulin-like growth factor 1 receptor	0.322	> 0.1	65.27	1.45	p < 0.01

This list includes genes that were found to pass the suggested threshold for genome-wide studies and candidate genes (shaded gray). Predicted gene sequences not shown. Igf1r was chosen due to its relationship with Insr. Grik4 was chosen based on previous work showing differentially methylation in other models. The corrected p-value, distance from TSS, and difference in methylation between MS and NH (corrected reads per million) are shown for each gene. Selected targets were also chosen for qPCR and their fold change (FC) and p-value is shown.

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