Genetic predisposition to high anxiety- and depression-like behavior coincides with diminished DNA methylation in the adult rat amygdala

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Abstract

Understanding biological mechanisms that shape vulnerability to emotional dysfunction is critical for elucidating the neurobiology of psychiatric illnesses like anxiety and depression. To elucidate molecular and epigenetic alterations in the brain that contribute to individual differences in emotionality, our laboratory utilized a rodent model of temperamental differences. Rats bred for low response to novelty (Low Responders, LRs) are inhibited in novel situations and display high anxiety, helplessness, and diminished sociability compared to High Novelty Responder (HR) rats. Our current transcriptome profiling experiment identified widespread gene expression differences in the amygdala of adult HR/LR rats; we hypothesize that HR/LR gene expression and downstream behavioral differences stem from distinct epigenetic (specifically DNA methylation) patterning in the HR/LR brain. Although we found similar levels of DNA methyltransferase proteins in the adult HR/LR amygdala, next-generation sequencing analysis of the methylome revealed 793 differentially methylated genomic sites between the groups. Most of the differentially methylated sites were hypermethylated in HR versus LR, so we next tested the hypothesis that enhancing DNA methylation in LRs would improve their anxiety/depression-like phenotype. We found that increasing DNA methylation in LRs (via increased dietary methyl donor content) improved their anxiety-like behavior and decreased their typically high levels of Forced Swim Test (FST) immobility; however, dietary methyl donor depletion exacerbated LRs' high FST immobility. These data are generally consistent with findings in depressed patients showing that treatment with DNA methylation-promoting agents improves depressive

symptoms, and highlight epigenetic mechanisms that may contribute to individual differences in risk for emotional dysfunction.

Introduction

Vulnerability to mental illness is associated with several factors including inborn differences in temperament and ability to cope with stress, which can be shaped through a combination of genetic and environmental influences [1-4]. Epigenetic mechanisms lie at the crossroads where “nature” meets “nurture” and likely mediate these gene × environment interactions long implicated in the pathogenesis of psychopathology [5-8]. A growing body of evidence indicates that abnormal DNA methylation and other epigenetic marks across the genome in post-mortem brain samples from psychiatric patients [9-16]. Naturally-occurring inter-individual epigenetic differences may trigger disease susceptibility and pathology [17-20], although early-life environmental conditions likely play an important role since they affect brain development and subsequent emotional behavior through epigenetic mechanisms [21-27]. Examining epigenetic abnormalities that contribute to the emergence of emotional dysfunction may shed light on the pathogenesis of psychiatric disorders. Moreover, since epigenetic processes are largely modifiable through pharmacological approaches or even dietary factors, epigenetic-targeted therapies offer attractive new treatment opportunities [28].

To study neurobiological (including epigenetic) factors that shape emotional behavior, our laboratory uses a rat model of temperamental differences. Sprague Dawley rats bred for low behavioral response to novelty (Low Responders, LRs) are inhibited in novel situations and display high fearfulness, anxiety, and diminished sociability and sexual motivation [29-32] compared to High Novelty Responder (HR) rats that are aggressive [33], impulsive, and prone to addictive behaviors [34]. The HR/LR

traits are heritable and emerge during early life [35], although they are also sensitive to stress exposure and variation in maternal care [36, 37]. We recently identified HR/LR differences in DNA methylation markers in the early postnatal limbic brain [38, 39]. A pivotal question now is: are DNA methylation differences present in adulthood and, if so, what roles do they play in shaping the HR/LR phenotypes? Moreover, although prior work indicates that hippocampal differences contribute to the disparate HR/LR behavioral phenotypes [35, 40-43], our recent data point to differences in the amygdala as well [36, 44]. The amygdala is well known for its roles in regulating novelty detection, emotion, and fear responses [45, 46], and clinical and basic animal studies alike indicate that individual differences in temperament correspond to distinct structure and function of the amygdala [47-52].

The present study examined gene expression and DNA methylation differences in the adult HR/LR amygdala that may contribute to their high baseline level of depression- and anxiety-like behavior. Given our previous observations of disparate gene expression and DNA methylation patterns in the early postnatal HR versus LR amygdala [38, 39, 44], we hypothesized that these differences would also occur during adulthood. To test this, we first performed a transcriptome profiling experiment in the amygdala of adult HR versus LR rats. We next examined DNA methylation in the HR versus LR amygdala by measuring global DNA methylation (5-methylcytosine) levels, DNA methyltransferase (DNMT) protein levels, and finally used next-generation sequencing to interrogate gene-specific methylation patterns. Because that analysis revealed a number of hypomethylated genomic sites in LR versus HR rats, our last experiment tested the hypothesis that enhancing DNA methylation in LR rats (by

increasing dietary methyl donor content) would improve aspects of their behavioral phenotype (effectively making them appear more “HR-like”). On the other hand, we predicted that decreasing DNA methylation in LRs (by depleting dietary methyl donor content) would exacerbate their high levels of anxiety and depression-like behavior.

Methods and Materials

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

2.1 Animals.

The animals used in this study were obtained from our in-house colony where the HR/LR bred lines were recently re-derived using a breeding strategy described previously in our original publication with the bred HR/LR rats [30]. The present experiments used HR/LR males from the 4th through 8th generations of our colony. All housing and testing facilities were maintained at 21-23°C and 50-55% humidity; rats were pair-housed in a 12:12 light-dark cycle (lights on/off at 6 AM/6 PM). To prevent possible litter effects, animals from our colony were derived from different litters within a given assay.

2.2 Transcriptome profiling in adult HR/LR amygdala.

Adult male HR and LR rats (n=5 per phenotype) were sacrificed by rapid decapitation to harvest brain tissue for genome-wide expression profiling. Brains were

removed, flash frozen in isopentane cooled to -30°C on dry ice, and then stored at -80°C until further use. 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 amygdala were then dissected and homogenized. RNA was isolated (NucleoSpin RNA II), quantified using a Nanodrop ND-1000 (Wilmington, DE), and stored at -80°C.

RNA samples from the adult HR/LR amygdala (n=5 samples per phenotype) were shipped on dry ice for transcriptome profiling using NimbleGen Rat Gene Expression 12x135 Arrays (26,419 target genes, 5 probes/targets; Arraystar, Rockville, MD) as previously described [53]. Briefly, RNA quality and quantity were assessed and total RNA was linearly amplified with Agilent's Low Input Quick Amp Kit (Agilent Technology). Double-stranded cDNA (ds-cDNA) was synthesized from the amplified cRNA using an Invitrogen SuperScript ds-cDNA synthesis kit in accordance with the NimbleGen Gene Expression Analysis protocol (NimbleGen Systems, Inc., USA). The microarray assay utilized an Axon GenePix 4000B microarray scanner (Molecular Devices Corporation). Scanned images were aligned and analyzed using NimbleScan software (version 2.5). Expression data were normalized through quantile normalization and the Robust Multichip Average (RMA) algorithm included in the NimbleScan software. Probe and gene level files were generated after normalization.

Subsequent data analysis was performed using Agilent GeneSpring GX software (version 12.6, Santa Clara, CA) and Metacore™ Data-Mining and Pathway Analysis software (Thomas Reuters, New York, NY). Probes with raw expression values less than 50 for any sample or any “predicted mRNA models” were excluded from analysis.

Following experimental grouping (i.e., HR/LR), expressed genes were considered statistically significant and included in downstream analysis if they displayed fold change >1.5, p-value < 0.05 after multiple test correction (Bonferroni family-wise error rate [FWER]).

To gain a better understanding of top molecular pathways that differed in the HR/LR amygdala, we used Metacore™ pathway analysis software (Thomas Reuters), considering genes with a fold change > 1.2, p-value of < 0.05. The pathway enrichment analysis was performed with these less stringent parameters to capture a broader scope of regulation with 2191 genes included in the analysis. Significantly enriched terms were identified using a Benjamini-Hochberg false discovery rate (BH) and p< 0.05. All genes discussed in this paper are referenced using the *Rattus norvegicus* gene symbol. The microarray expression data will be made publically available via the gene expression omnibus (GEO) database with the accession number: GSE88874.

2.3 DNA methyltransferase (DNMT) protein expression and global DNA methylation (5-methylcytosine) levels and in adult HR vs. LR amygdala.

To examine protein expression levels for three primary DNMTs – DNMT1, DNMT3a, and DNMT3b – in the amygdala of adult male HR and LR rats. Amygdala tissue punches were collected from HR/LR rats (n=8/group) to isolate protein (EpiQuik Nuclear Extraction kit, Epigentek, Farmingdale, NY, catalog # OP-0002-1). Extracted protein concentration was quantified using a BCA protein assay (Thermo Scientific, Watham, MA), and samples were used in EpiQuik DNMT1, DNMT3a and DNMT3b

protein assays (Epigentek, catalog # P-3011, P-3012, P-3013, respectively) according to manufacturer instructions.

A separate set of HR/LR amygdala tissue punches were collected to isolate DNA using a DNeasy extraction kit (Hilden, Germany; n=8/group). DNA samples were quantified using a Nanodrop ND-1000 (Wilmington, DE), stored at -20°C, and later used to assess levels of 5-methylcytosine. The Epigentek MethylFlash Methylated DNA Quantification Kit (Colorimetric) measured global DNA methylation (5-methylcytosine) per manufacturer instructions and as we previously described [54]. 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.

2.4 Methylated DNA capture coupled with next-generation sequencing (MethylCap-Seq) to map the methylome in adult HR versus LR amygdala.

To survey the methylome, 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 HR/LR amygdala 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 [55]. Methylated DNA fragments were captured with a methyl-binding domain 2 (MBD2) protein coupled to paramagnetic Dynabeads® M-280 Streptavidin per

manufacturer instructions. 1µg of sonicated DNA was used, and captured fragments were eluted using 3500mM NaCl. Captured material was purified using QIAquick PCR purification spin columns (Qiagen, Valencia, CA) and quantified using Quant-it high sensitivity DNA Assay Kit (Invitrogen, Grand Island, NY, Q-33120) and an Agilent 2100 Bioanalyzer high sensitivity chip kit (Agilent Technologies, Santa Clara, CA). This sequencing requires 100 ng input material, which necessitated pooling samples; as such, we pooled two animals to create each sequencing sample, yielding n=3 samples per HR or LR phenotype for sequencing analysis. 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). Afterward, 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 three biological replicates per group 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 [56-58]. Raw single-end sequenced reads were quality controlled and filtered for read quality (FastQC, Galaxy). Sample reads were mapped onto sequence Rn5 assembly using Bowtie for Illumina (http://bowtie-bio.sourceforge.net/index.shtml). Overall we obtained an average of ~11.5M mapped single-end reads from MethylCap-seq samples. The BAM files have been made publicly available on NCBI's SRA under accession: PRJNA344780. 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 [59]. 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 [60]. Statistical analysis compared methylation peak intensity differences (MIDs) between HR and LR samples (p-value of 0.05 with BH multiple test corrections) across the genome to evaluated distinct HR vs. LR 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 promoters of the TSS.

2.5 Quantitative real-time PCR (qRT-PCR) confirmation of select genes.

In order to confirm that DNA methylation differences detected by MethylCap-seq lead to predicted mRNA expression changes within select genes, we conducted 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 three genes: *Grin2b* (Rn00680474_m1; RefSeq: NM_012574.1), *Pclo* (Rn00571796_m1; RefSeq: NM_001110797.1) and *Stx1b* (Rn01510167_m1; RefSeq: NM_012700.2) and used *Abl1* (Rn01436239_m1; NM_001100850) as a housekeeping gene. RNA was isolated (NucleoSpin RNA II) from amygdalar samples collected from adult HR and LR rats (n=5 per condition). 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 for each sample. Relative fold changes between HR/LR groups were compared for a given gene at a particular time point were calculated using the ΔΔCT method.

2.6 Evaluating the impact of methyl donor dietary content on adult LR rats' behavior.

In order to begin to assess the functional implications of HR/LR DNA methylation differences, we examined the effects of manipulating methyl donor content in the diet of adult LR males by feeding them either a control diet, one that was depleted of methyl donors, or a diet supplemented with high levels of methyl donors. Thus, LR male rats obtained from our in-house colony were fed standard rat chow (NIH-31 Open Formula 7917 18% protein diet, Harlan Laboratories, Oxon, UK) *ad libitum* until they reached postnatal day (P) 75. On P75, LR males were assigned to one of three diet conditions to receive: *(1)* control diet (LR-CON); *(2)* chow that was 90% depleted of methyl donors (LR-DEP); or *(3)* a methyl supplemented diet (LR-SUP; n=16 LR males per group). The LR-CON animals received commercially made semisynthetic L-amino acid-complete rodent diet no. A10021 (Research Diets Inc, New Brunkswick, NJ). The LR-DEP group received a L-amino acid-defined rodent diet lacking 90% of normal requirements of choline, folate, and methionine (diet no. A04062402, Research Diets Inc, New Brunkswick, NJ), and the LR-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).

In this experiment, all diet groups were food-restricted to receive 35 g/cage/day in the first three weeks, and then increased to 40 g/cage/day during the 2-week period

of behavioral testing. Each cage housed 2 rats. Diet restriction was done to ensure equal feeding between groups since previous studies had reported diminished food intake in rodents receiving a methyl-depleted diet [61, 62]. Animals were weighed each week starting on the first day of diet exposure. There were no significant differences in the weight of cage mates (2-way ANOVA, $p > 0.05$ for cage mates within each group). See supplementary figure of weights during treatment for details (**Sup. Fig. 1**).

2.6.1 Behavioral test battery.

After receiving the control, methyl-depleted, or methyl-supplemented diets for three weeks, LR male rats were evaluated in a behavioral test battery comprised of several classic rodent tests of anxiety- and depression-related behavior: Elevated Plus Maze (EPM), Open Field Test (OFT), Social Interaction (SI) test, and Forced Swim Test (FST). All animals were subjected to the full test battery in this test order, with 1-2 days' rest between tests. All tests were performed between 8 AM-12:30 PM, conducted under dim lighting (30 lux) and sessions were videotaped and analyzed by Ethovision® XT 8.0 videotracking software (Noldus, Wageningen, The Netherlands). As the light phase for the animals is 6AM- 6PM, the testing takes place early in the light phase, which is when rodents exhibit the nadir of their daily corticosterone level. Our previous study examining the circadian cycle differences in HR/LR showed that during 8 AM -12:30 PM there are no difference in baseline corticosterone levels or home cage activity [63].

The EPM apparatus was constructed of black Plexiglas™ with four elevated arms (70 cm from the floor, 45 cm long, 12 cm wide) arranged in a cross. Two opposite arms were open (with lighting at 30 lux) and the other two arms were enclosed by 45-

cm-high walls, which limited lighting to 3-5 lux. At the start of each test, a rat was placed in the center square of the EPM facing a closed arm, then allowed to freely explore for 5 min. We monitored the following: latency to enter the open arms; frequency to visit, time spent within and distance traveled in the open arms, closed arms and center square.

The OFT 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 OF, the amount of time spent and distance traveled in the center, periphery, and corners of the apparatus were quantified. A trained observer that was blinded to experimental groups manually assessed grooming and rearing behavior using a computerized system provided in the software.

The SI test was performed in 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 of two 10-min sessions. On day 1, 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. On day 2, the experimental 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. We analyzed the latency for experimental animals to enter chambers containing social stimulus rats as well as number of visits and time spent in each chamber.

Porsolt's FST was performed as we previously described [64] with 30-cm deep 25°C water in Plexiglas™ containers (45 cm high x 20 cm diameter). On FST day 1, rats were placed into the water for 15-min; 24 h later, the rats were tested for another 5 min. 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 as a classical indicator of behavioral despair and depressive-like behavior [65].

2.6.2 Tissue collection in diet-manipulated LR males.

One day after the final behavioral test, a subset of the LR-CON, LR-DEP, and LR-SUP condition LR males (n=8/group) were sacrificed by rapid decapitation to collect brain and liver, which were removed, flash frozen in isopentane cooled to -30°C on dry ice, and then stored at -80°C until further processing.

2.8 Statistical Analysis.

Data analyses for the microarray experiment and MethylCap-seq experiment are described above. Data from the qRT-PCR, DNMT protein and 5-methylcytosine 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.

Comparisons of HR and LR rats were analyzed by a student t-test (or non-parametric equivalent when necessary). In the diet manipulation studies, rats' weight was analyzed by two-way ANOVA, with age and diet condition as independent variables. In the diet manipulation study, behavioral data were analyzed by one-way ANOVA to examine differences among the LR-CON, LR-DEP, and LR-SUP LR rats in the EPM, OFT, and FST.

Results

3.1 Transcriptome profiling in the adult HR versus LR amygdala.

Based on the dramatic HR/LR transcriptome differences in the early postnatal amygdala and hippocampus [35, 44], we sought to determine whether these alterations exist into adulthood. We therefore performed a transcriptome profiling study in the adult HR and LR amygdala. This showed that more than 2000 genes were differentially expressed in the HR versus LR adult amygdala, with the majority of the genes up-regulated in HR compared to LR ($p < 0.05$, BH, Fold Change > 1.5; **Fig. 1A, C**). Through Metacore™ pathway maps analysis, we found that synaptic protein recycling and GABA receptor processes were among the top regulated pathways (**Fig 1B**; $p < 0.05$ after BH correction). Of the top ten regulated pathways, GABA-B receptor signaling in presynaptic nerve terminals, Gastin in cell growth and proliferation, and ACM (acetylcholine receptors, muscarinic) regulation of nerve impulse were the only pathways enriched in genes downregulated in HR. The other pathways were enriched with genes that were upregulated in HR vs. LR amygdala (regulation of individual genes not shown).

3.2 Adult HR/LR rats show similar levels of DNMT protein expression and global 5-methylcytosine in the amygdala.

Given the marked HR/LR transcriptome differences in the adult amygdala, we wanted to begin to examine possible epigenetic mechanisms that may underlie these differences. Because previous studies found evidence of DNA methylation alterations in the early postnatal HR/LR brain [38, 39], we first measured global DNA methylation (5-methylcytosine) levels and DNMT protein levels. We found no HR/LR differences in global DNA methylation (5-methylcytosine) levels (**Fig. 1D**), and no significant differences in DNMT1, -3a, or -3b expression (**Fig. 1E**).

3.3 Next-generation sequencing analysis revealed disparate methylome landscape and gene-specific DNA methylation patterns in the adult HR versus LR amygdala.

While no global differences in DNA methylation or DNMTs were found, local changes only within the epigenetic status surrounding genes can regulate cellular function. To delve more deeply into possible HR/LR DNA methylation differences in the amygdala, we utilized next-generation sequencing technology to map and compare the methylome of the HR versus LR adult amygdala. We began by using MACS peak calling to examine total number of differentially methylated regions (DMRs). The MethylCap-Seq methodology allowed us to sequence methylated DNA fragments with the resolution of a few hundred bases pairs. The resulting data were filtered in two major ways. First, we applied stringent statistical criteria to the data. A Manhattan plot illustrates DMRs across the genome with 181 sites that were differentially methylated in

the HR/LR adult amygdala (p-value < 1 x 10^{-5}, the suggested threshold in genome-wide studies, indicated by a blue line in **Fig. 2**). This group of DMRs included 47 gene regulatory sites (a region within the gene body or up to 10 kb upstream of a gene). Next we applied the genome-wide association significance threshold (p-value < 5 x 10^{-8}; shown as a red line in **Fig. 2**) to set even more highly conservative analytical parameters and found 118 DMRs within 26 gene regulatory sites. Overall, our analysis identified 35 genes that contain DMRs in regulatory sites, with some genes displaying multiple DMRs. Some example genes are displayed in plots in Figure 2. For instance, we identified two DMRs within intronic regions of *Farp1* (FERM, RhoGEF and pleckstrin domain-containing protein 1), a cytoskeleton protein that has be implicated in regulation of synaptic organization [66]. Other example genes that showed HR/LR methylation differences include: *Prkca* (protein kinase Cα), *Prkd1* (protein kinase D1), and DNA binding proteins *Zfp575* and *Taf1* (**Fig 2**, p < 1 x 10^{-5}).

Next, to examine possible methylation patterns and pathway analysis, we adjusted the threshold to allow more liberal measures (p < 0.05 with BH correction in MID comparison after MACS peak calling) to decrease our chance of false negative results and found 793 total DMRs with these parameters. Using this data set, we evaluated the number of hypermethylated and hypomethylated genomic regions in the HR versus LR amygdala broken down by genomic location (**Fig. 3A-B**). Independent of genomic location, there is a higher proportion of HR/LR hypermethylated than those hypomethylated (**Fig 3B**). Most of the DMRs occur in intergenic sites though over a quarter of the sites are within the gene body **(Fig.3A**). Using the genes with DMRs within the gene body or 2 kb upstream of the gene (here, referred to as promoters), we

performed KEGG analysis to find functional pathways enriched with genes that displayed altered DNA methylation in HR versus LR (**Fig. 3C**; p< 0.05 with BH correction, minimum of 3 genes within a category). There were 107 genes with associated DMRs included in this analysis and yielded 4 enriched KEGG pathways: neuroactive ligand, Huntington's disease, metabolic pathways and MAPK signaling.

3.4 Relationship between methylome and transcriptome differences in the adult HR vs. LR amygdala.

As another analysis tactic, we cross-referenced results of our HR/LR adult amygdala transcriptome profiling experiment (described in *Section 3.1*) and the MethylCap-seq study to identify genes that were differentially expressed in HR/LR adult amygdala and also showed methylation differences through MACS peak calling in the gene promoter or gene body (**Fig. 3D**). Consequently, the expression levels of 2191 transcripts were used to identify genes that displayed both differential mRNA expression as well as differential methylation marks in the HR versus LR amygdala. Of the 107 DMRs within the gene body or within 2kb upstream of the TSS, 16 genes were found to be differentially expressed in HR/LR amygdala in our microarray study (**Table 1**). Three of the genes (glutamate receptor subunit epsilon-2 [*Grin2b],* piccolo presynaptic cytomatrix protein isoform 1 [*Pclo])* and syntaxin-1B *[Stx1]))* exhibited greater transcript expression in HR versus LR adult amygdala (*Grin2b:* adjusted p<0.001, Fold Change (FC) = 4.66; *Pclo: adjusted p< 0.001, FC = 2.017; Stx1b*: adjusted p<0.01, FC = 2.00; Table 1). We then confirmed these results with qPCR (*Grin2b*: FC=1.57; t= 3.5413, df=

8, p= 0.0076; *Pclo*: FC= 1.42, t= 2.348; df=8, p= 0.046; *Stx1b*: FC= 1.57, t=4.2063, df=8, p= 0.003; **Table 1**).

Our next-generation sequencing analysis revealed decreased DNA methylation in HR versus LR within the gene body of *Grin2b* (p= 0.01, MID= -0.55), a regulatory subunit of NMDA glutamate receptors. *Pclo*, which serves as a scaffolding in the presynaptic active zone, showed increased DNA methylation within promoters of the gene body (p< 0.001, MID= 0.831). *Stx1b,* another presynaptic protein that has been shown to regulate GABA and glutamate synaptic vesicles release [67], showed increased methylation in HR versus LR across the gene body (adjusted p<0.001, MID= 0.644; **Fig. 3D**).

3.5 Effects of manipulating dietary methyl donor content in adult male LR rats.

In our MethylCap-seq study, we found that most DMRs were hypermethylated (69% of all DMRs and 64.5% of those in promoter or gene body) in HR/LR amygdala, so we hypothesized that increasing DNA methylation in adult LRs would lead to an improvement of their anxiety/depression-like behavior. To examine the functional implications of LRs' diminished DNA methylation levels in the brain (relative to HRs), we manipulated dietary methyl donor content in diet of adult LR males (either depleting or supplementing methyl donor content) and tested its effect on several anxiety- and depression-like behaviors.

3.5.1 Adult male LR weight during methyl donor dietary manipulation.

All rats gained weight during the diet manipulation period (effect of time [$F(4, 180)= 28.87$, $p<0.0001$]), and there was no significant effect of diet (**Sup. Figure 1**). At the conclusion of the diet manipulation and behavioral assessment period, we assessed 5-methylcytosine levels in liver tissue of LR-CON, LR-DEP, and LR-SUP rats to determine the effect of methyl donor deficient or supplemented diet on systemic DNA methylation levels. There was a main effect of diet treatment on global DNA methylation levels ($F=59.74$, $p<0.006$). Post hoc analysis revealed that 5-methylcytosine levels were significantly increased in LR-SUP versus LR-CON and LR-DEP groups (LR-SUP 0.21 ± 0.05 versus LR-CON 0.14 ± 0.03; LR-DEP 0.16 ± 0.04; 5-methylcytosine as percentage of total DNA).

3.5.2 Dietary methyl donor depletion worsened LRs' anxiety- and depression-like behavior, while methyl donor supplementation improved it.

Adult LR males that were subjected to each diet group (LR-CON, LR-DEP, and LR-SUP diets) were evaluated in multiple tests of anxiety- and depression-like behavior. Because our MethylCap-Seq findings suggested that LRs have lower DNA methylation levels in the brain compared to HRs, we hypothesized that: ***a)*** increasing DNA methylation levels in adult LRs via methyl donor-supplementation would lead to improved anxiety and/or depression-like behavior; whereas ***b)*** methyl donor depletion would worsen these aspects of their behavior. In the EPM, we found that methyl donor supplementation had an anxiolytic effect (main effect of diet on time spent in the anxiogenic open arms; Kruskal-Wallis statistic= 9.092, $p=0.01$; **Fig. 4A**, left). Methyl donor supplemented LRs (LR-SUP) spent more time in open arms than LR-CON and

LR-DEP groups (mean rank differences= 10.22 and 9.522, respectively, $p<0.05$ for each comparison). The LR-SUP rats' high level of open arm exploration was not due a general increase in locomotor activity, since all three treatment groups traversed a similar distance during the 5-min EPM test (**Fig.4A**, right).

In the OFT, the LR-CON, LR-DEP, and LR--SUP groups spent similarly low amounts of time in the center of the OF, and the groups showed similar level of activity in the novel environment (**Fig. 4B**). In the SI test, methyl donor supplementation subtly improved LRs' typically low levels of SI. LR-SUP animals showed a shorter latency to explore the novel stimulus rat (**Fig. 4C**, left panel; main effect of diet [$F(2, 90)=4.39$, $p=0.0152$]; post hoc analysis showed that LR--SUP differed from LR-CON and LR-DEP groups, $p<0.05$). Although LR--SUP animals initiate social interaction more quickly, they spent similar amounts of time in close proximity to the social stimuli compared to the LR-CON and LR-DEP groups (**Fig. 4C**, right).

In the FST, we found that methyl donor dietary depletion exacerbated LR rats' already high levels of FST immobility (main effect of diet [$F=11.94$, $p<0.0001$]), with post hoc analysis showing that LR-DEP rats spent more time immobile compared to LR-CON and LR-SUP groups ($p<0.05$ for each comparison). Interestingly, methyl donor supplementation, on the other hand, showed a trend for an antidepressant effect by improving FST immobility ($p=0.09$ for LR-CON versus LR-SUP comparison; **Fig. 4D**).

Discussion

There is great interest in uncovering epigenetic abnormalities involved in the neurobiology of emotional disorders. Because epigenetic mechanisms (such as DNA

methylation) simultaneously regulate myriad genes, a perturbation of a regulatory system such as DNA methylation could induce many gene expression changes and downstream biobehavioral effects. Recent work in our laboratory identified DNA methylation differences as well as widespread transcriptome differences in the limbic brains of LR/HR rats during early postnatal development [38, 39, 44]. We hypothesized that these differences would also occur in adulthood and that HR/LR DNA methylation differences represent a molecular mechanism that drives their disparate emotional behavior phenotypes. The present transcriptome study revealed a host of gene expression differences in the adult HR/LR amygdala, including genes involved in synaptic activity. Methylome analysis revealed several hundred differentially methylated sites in the adult LR/HR amygdala, including many regions that regulate gene transcription. Of particular interest were genes that we found to be both differentially methylated and differentially expressed (based on microarray results). These included *cacna1a, grin2b, and kcnip2*, which are all ionotropic channels or receptor subunits that play a role in synaptic transmission; and *pclo, stx1b,* and *syt17*, which each regulate synaptic function. To begin to assess the functional importance of HR/LR DNA methylation differences, our final study manipulated dietary methyl donor content in adult male LR rats to test whether promoting or hindering DNA methylation would influence LRs' typically high levels of anxiety- and/or depression-like behavior. We showed that supplementing adult LR males' diet with extra methyl donors subtly decreased their anxiety-like behavior in the EPM and SI test, and showed a trend towards decreasing their typically high levels of depression-like behavior (immobility) in the FST. Dietary methyl depletion, on the other hand, exacerbated LRs' FST

depression-like behavior, leading to significantly higher immobility levels compared to the control and methyl supplemented LR groups.

4.1 HR/LR gene expression differences in the amygdala.

Prior studies using rodent models relevant to emotional disorders have found broad gene expression alterations in the adult amygdala. Widespread transcript changes have been reported in the adult amygdala of animals exposed to unpredictable chronic mild stress [68], chronic restraint stress [69, 70], or maternal separation [71]. Gene expression differences have been found in the amygdala of mice and rats that naturally exhibit high versus low anxiety/depression-like behavior [69, 70, 72]. Data from these studies, coupled with our current findings, highlight complex molecular changes in the amygdala that likely contribute to individual differences in emotional behavior. Although broad functional classes of genes are similarly affected across studies, there is little overlap in specific genes identified. This may be due to technical issues related to microarrays. Another possibility, though, is that distinct molecular signatures go along with different types of chronic stress or inborn predispositions for high anxiety- or depression-like behavior [68, 70].

4.2 DNA methylation differences in the adult HR versus LR amygdala.

Post-mortem brain samples from psychiatric patients show signs of abnormal DNA methylation and other epigenetic aberrations [9-16]. Environmental factors and life experience modify epigenetic processes in brain, which may contribute to the

pathophysiology of mental illness [21-27], but naturally-occurring inter-individual epigenetic differences may also trigger disease susceptibility and pathology [17-20]. Thus, it is unknown whether epigenetic abnormalities present in psychiatric disorders ***(a)*** represent a primary deficit driving the disease; ***(b)*** whether changes are secondary to environmental or experiential factors (e.g., exposure to chronic stress), or ***(c)*** a combination of both. Human twin studies point to a high degree of heritability of epigenetic marks (including methylation) [16, 73], although other work in humans [17, 19, 74] and animals [75-77] demonstrates considerable inter-individual variation in DNA methylation of CpG islands and other genomic regions. The functional implications of these inter-individual epigenetic differences are unknown, but it is intriguing to consider how such differences may impact brain development, function, and susceptibility to disease. Animal models of emotional dysfunction (such as our bred HR/LR model) can then serve as a powerful tool to carefully dissect some of these factors to better inform future human studies.

Through MethylCap-seq, we found 793 DMRs in HR/LR amygdala samples, with most of the sites hypermethylated. Many of the DMRs occurred within intergenic sites; it is difficult to decipher the functional import of these changes since the local genetic and epigenetic landscape can interact with such differences to shape downstream effects. For instance, differential methylation within intergenic regions could lead to structural changes like chromatin remodeling or could regulate transcription through *trans* sites, athough additional experiments would be required to explore these possibilities. We found that many of the genes that were differentially methylated in HR/LR rats were involved in neuroactive ligand-receptor interaction, metabolic pathways, and synaptic

activity. We selected a subset of these genes to confirm HR/LR gene expression differences, focusing on genes that have previously been implicated in mood disorders and animal models of stress and emotional dysfunction: NMDA glutamate receptor subunit NR2B (*Grin2b*), and presynaptic proteins *Pclo* and *Stx1b*.

Previous work found that *Grin2b* expression positively correlated with high levels of anxiety-like behavior in Wistar rats [78] and mice [62], and was also disrupted in humans that suffered depression and committed suicide [79, 80]. Our *Grin2b* expression results conflict with these earlier findings (since we found lower expression in high anxiety/depression-like behavior prone LRs), although such discrepancies may be related to different rodent strains used and/or the fact that the other work was done in stressed animals while our tissue was collected from unstressed rats. We also found reduced expression presynaptic-related genes *Pclo* and *Stx1b* in the LR versus HR amygdala. Loss of Pclo protein has been shown to leads to synapse degradation [81] and reduced long-term potentiation [82]. Altered Pclo protein levels have been reported in the amygdala of patients suffering schizophrenia [83], and other work found an association between a single nucleotide polymorphism within *Pclo* (rs2522833) and aberrant amygdalar activation during fear conditioning and cognitive tasks [84, 85]. These later results suggested that diminished Pclo function in the amygdala may be associated with high levels of fear and anxiety, which would be consistent with our findings in high anxiety-prone LR rats. To our knowledge, our finding of an association between *Stx1b* and high levels of anxiety/depression-like behavior has not been reported before.

4.3 Increasing dietary methyl donor content leads to improved anxiety- and depressive-like behavior in LR rats.

Diet is an important environmental factor that has the capacity to influence DNA methylation since dietary folate, choline, and methionine act as methyl donors for one-carbon transfer reactions like DNA methylation. DNMTs transfer methyl groups from S-adenosylmethionine (SAM) to cytosine [86], so diets lacking folate or other methyl donors can impede SAM synthesis, thereby leading to DNA hypomethylation [87-89]. Depleting dietary methyl donor content has been shown to decrease DNA methylation markers in the brain [89], and impair fear memory [62, 90]. On the other hand, boosting levels of methyl donors (or SAM itself) increases DNA methylation levels in brain [91], and elicits antidepressant effects in rats and mice, such as decreased immobility in the FST and improved stress-induced anhedonia [92-95]. Since our methylome profiling data revealed a greater number of hypomethylated genomic regions in brain tissue from adult LR vs. HR rats, we hypothesized that manipulating DNA methylation in LR rats (by altering dietary methyl donor content) could influence aspects of their behavioral phenotype. Specifically we predicted: (***a)*** that supplementing methyl donors in LR rats' diet (which has been shown to enhance methylation levels in brain [91]) would have antidepressant effects, whereas ***(b)*** depleting dietary methyl donors (which would lower DNA methylation levels in the brain [89]) would exacerbate LRs' anxiety/depression-like phenotype. We found that, indeed, depleting dietary methyl donor content worsened LRs' depression-like behavior (immobility) in the FST. On the other hand, feeding LR rats a diet enriched with methyl donors subtly improved their anxiety-like behavior and showed a trend for improving their FST immobility. These data are generally consistent

with clinical findings in depressed patients showing that treatment with agents that promote DNA methylation (i.e. S-adenosyl-L-methionine (SAMe) [96-98] or L-methylfolate [99-101]) improves depressive symptoms. Important next steps will be to determine whether the dietary methyl donor supplementation elicited its behavioral effects through methylation changes in specific genes and whether those affected genes are similar to ones found to be differentially methylated in baseline HR/LR amygdala.

Results from other rodent studies using diet or pharmacological approaches to manipulate DNA methylation have produced variable behavioral results. For instance, studies in mice found that chronic dietary methyl depletion interfered with contextual fear conditioning/memory, but did not impact anxiety-like behavior [62, 90, 102]. A study in Wistar rats combined methyl diet deficiency with chronic unpredictable stress and found that methyl donor deficiency improved chronic stress-elicited anxiety-like behavior, but did not affect FST immobility [103]. Studies in mice [92] and rats [93] reported that chronic treatment with methylation promoting agents (folic acid or S-adenosyl-L-methionine) had antidepressant and anxiolytic effects. Intracranial L-methionine infusion increased anxiety-like behavior [104] and FST immobility [105], although this effect occurred specifically in animals that typically show low basal anxiety/depression-like behavior (due to receiving high levels of maternal care in infancy); rats that typically show high basal anxiety/depression-like behavior (due to receiving low levels of maternal care) were unaffected by L-methionine treatment. Together these disparate findings suggest that either boosting or lowering methylation levels in the brain can elicit varied effects depending upon the species (i.e. mouse or

rat) being tested, certain experimental conditions (i.e. under chronic stress conditions), as well as individual differences in baseline emotionality and stress reactivity.

4.4 Technical considerations.

An important limitation of the present study is the fact that our experiments used only male rats. There is a relative lack of information about epigenetic differences in the brains of males versus females [106, 107] and how such differences may contribute to sexually dimorphic risk for emotional dysfunction [108]. Thus, it will be important for future studies to determine whether both male and female individuals with high versus low propensity to an anxiety/depression-like phenotype display similar or unique differences in the limbic transcriptome and how those differences may impact behavioral outcomes. We have conducted some studies in HR/LR females and found that they generally display similar behavioral phenotypes compared to their male counterparts [30, 109, 110]. Future experiments will plan to determine whether HR/LR females display similar neural and epigenetic differences as what has been found in HR/LR males, and whether methyl donor depletion and/or supplementation influences LR females as it does in males.

A caveat of our transcriptome and methylome studies in the HR versus LR amygdala is that our dissection potentially included multiple amygdalar subnuclei, which are characterized by unique functions and anatomical connections. It would have been ideal if we could have microdissected specific amygdalar subnuclei for these studies since it could potentially offer the chance to identify distinctly altered molecular processes within each subregion. Our primary obstacle was the fairly high amount of

raw material needed for the molecular studies, particularly the MethylCap-Seq procedure. As technologies improve, we hope to be able to sequence DNA and RNA not only from distinct subnuclei, but ideally distinct cell populations.

For this study, we chose to use a diet manipulation rather than a more invasive approach to reduce the amount of stress and handling the animals experience before behavior testing. Though, a caveat to utilizing such a tool is loss of specificity. Future studies will utilize intracranial injections of siRNA of target proteins such as Grin2b, Pclo or Stx1b into the amygdala of adult HR/LR rats to assess their role in emotionality. Another limitation of the methyl donor diet manipulation experiment is that it was only conducted in LR rats, not HRs. We hypothesized that LRs' deficient methylation levels (relative to HRs) may contribute to their high baseline anxiety- and depression-like behaviors and therefore sought to test whether increasing their methylation via dietary methyl donor supplementation would improve aspects of their behavior. It would be interesting to repeat the methyl donor diet manipulation in HR rats to determine whether they would show a similar (or disparate) reaction to the diet manipulation. Another useful control for this experiment could have been inclusion of a group that exhibits a 'normative' phenotype that is intermediate relative to the extreme HR and LR phenotypes. Newer experiments in our laboratory have begun to incorporate an "Intermediate Responder" (IR) group, which is created by cross-breeding the HR/LR lines; IR rats display an intermediate level of novelty-induced activity, anxiety- and depression-like behavioral measures relative to the extreme HR and LR responses [34].

4.5 Conclusions.

In summary, previous work in our laboratory has demonstrated widespread transcriptome differences in the limbic brains of rats that grow to display a high versus low propensity to anxiety and depression-like behavior [35, 44], and more recent findings suggest that these differences stem from epigenetic differences [38]. These results suggest that these differences exist in adulthood and that enhanced DNA methylation levels in adult LR rats (via dietary methyl donor supplementation), improved aspects of their anxiety/depression-like behavioral phenotype. Future experiments will continue to interrogate specific molecular processes that are altered in HR/LR rats and may contribute to their behavioral phenotypes to better understand how epigenetic mechanisms like DNA methylation contribute to individual differences in temperament and risk for emotional dysfunction.

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Figure legends

Figure 1. ***Transcriptome profiling and global DNA methylation in the adult HR/LR amygdala.*** A, Heatmap illustrating gene expression differences in the adult male HR/LR amygdala. B, Metacore Pathway Maps analysis identified the top ten pathways altered in the adult HR/LR amygdala with their associated –log p-values (represented by blue bars) and enrichment ratio (red bars; which compares number of altered genes to number of genes within the pathway). C, Number of gene down- or up-regulated in HR compared to LR ($p < 0.05$, BH, Fold Change > 1.5). D, 5 methylcytosine levels between HR/LR in the amygdala. E, Levels of DNA methyltransferase (DNMT)-1, -3a, and -3b in adult HR/LR amygdala. No significant differences found in HR/LR amygdala in global DNA methylation or DNMT proteins ($p > 0.05$).

Figure 2. ***Manhattan plot illustrating differentially methylated sites in the adult HR versus LR amygdala*****.** Differentially methylated regions (DMRs) are shown by chromosome (along the x-axis) and the negative logarithm of the association p-value (–log10 (p-value) on the y-axis). There were 181 DMRs in the HR/LR adult amygdala (p-value < 1 x 10-5, the suggested threshold in genome-wide studies, indicated by a blue line). This group of DMRs included 47 gene regulatory sites (a region within the gene body or gene promoters). When applying even more conservative analytical parameters (genome-wide association significance threshold p-value < 5 x 10-8, indicated by a red line), we found 118 DMRs within 26 gene regulatory sites. Overall, our analysis identified 35 genes containing DMRs in regulatory sites, with some genes displaying multiple DMRs. Genes with known function that met these criteria are labelled.

Figure 3. ***HR/LR rats display a number of DNA methylation differences in the amygdala within several genomic regions*****. A,** Distribution of DMRs within different genomic regions in the HR/LR amygdala filtering for MIDs $p < 0.05$ with BH correction. **B,** The number of hypermethylated (red bar) and hypomethylated (blue bar) sites within different genomic regions. **C**, KEGG analysis of genes that contain DMRs within the gene body or promoter of the gene. **D,** By cross-referencing results of our HR/LR adult amygdala transcriptome profiling experiment and the next-generation sequencing methylome analysis, we identified 16 genes that were differentially expressed and also contained DMRs in the gene promoter or gene body.

Figure 4. ***Manipulating dietary methyl donor content influenced adult male LRs' anxiety and depression-like behavior.*** **A**, In EPM, LR rats that received a methyl-donor supplemented diet (LR-SUP) spent significantly more time in the Open Arms compared to LRs receiving either control diet (LR-CON) or a methyl donor depleted diet (LR-DEP). All three groups displayed similar activity levels during the 5-min test**. B,** In OFT, all LR groups spent similarly low amounts of time in the center of the open field, and displayed similar activity levels. **C**, During SI testing, we assessed experimental rats' latency to visit a novel female or male stimulus rat (left) as well as total amount of time spent in a chamber with a novel male or female stimulus rat, or empty neutral zone. Dietary methyl content did not impact LRs' social behavior in this task. **D**, In FST, dietary methyl donor content influenced LRs' immobility (an indicator of behavioral despair). LR-DEP showed significantly greater immobility compared to LR-CON and LR-

SUP groups, suggesting that methyl depletion worsened LRs' already high levels of depression-like behavior. On the other hand, the LR-SUP LR group showed a trend towards reduced FST immobility compared to LR-CON LR males, suggesting that a methyl-rich diet exerts antidepressant effects. Data represent mean ± SEM; * indicates p-value < 0.05 compared to the other groups. + indicates a non-significant trend with p-value= 0.09.

Supplementary Figure 1. ***The weights of LR adult males during methyl donor diet feeding*****.** Before receiving the methyl donor, the animals received standard chow. At week 0, the animals were randomly assigned to a diet group. Behavioral testing took place at week 4. Analysis through 2-way ANOVA showed an effect of time [$F(4, 180)= 28.87$, $p<0.0001$]), but there was no significant effect of diet.

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