Title

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Authors

Glover, ME
Cohen, JL
Singer, JR
et al.

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Examining the role of microbiota in emotional behavior: antibiotic treatment exacerbates anxiety in high anxiety-prone male rats.

ME Glover[a,§], **JL Cohen**[b], **JR Singer**[c], **MN Sabbagh**[a], **JR Rainville**[a], **MT Hyland**[a], **CD Morrow**[d], **CT Weaver**[e], **GE Hodes**[a], **A. Kerman Ilan**[a,f], **SM Clinton**[a]

[a]School of Neuroscience, Virginia Polytechnic Institute and State University, Blacksburg VA, USA

[b]Department of Psychiatry, University of California, San Francisco, CA, USA

[c]MD/PhD Medical Scientist Training Program, University of Alabama-Birmingham, Birmingham AL, USA

[d]Department of Cell, Developmental, and Integrative Biology, University of Alabama-Birmingham, Birmingham, AL, USA

[e]Department of Pathology, University of Alabama-Birmingham, Birmingham, AL, USA

[f]Behavioral Health Service Line, Veterans Affairs Pittsburgh Health System, Pittsburgh, PA, USA

Abstract

Intestinal microbiota are essential for healthy gastrointestinal function and also broadly influence brain function and behavior, in part, through changes in immune function. Gastrointestinal disorders are highly comorbid with psychiatric disorders, although biological mechanisms linking these disorders are poorly understood. The present study utilized rats bred for distinct emotional behavior phenotypes to examine relationships between emotionality, the microbiome, and immune markers. Prior work showed that Low Novelty Responder (LR) rats exhibit high levels of anxiety- and depression-related behaviors as well as myriad neurobiological differences compared to High Novelty Responders (HRs). Here, we hypothesized that the divergent HR/LR phenotypes are accompanied by changes in fecal microbiome composition. We used next-generation sequencing to assess the HR/LR microbiomes and then treated adult HR/LR males with an antibiotic cocktail to test whether it altered behavior. Given known connections between the microbiome and immune system, we also analyzed circulating cytokines and metabolic factors to determine relationships between peripheral immune markers, gut microbiome components, and behavioral measures. There were no baseline HR/LR microbiome differences, and antibiotic treatment disrupted the microbiome in both HR and LR rats. Antibiotic treatment exacerbated aspects of HR/LR behavior, increasing LRs' already high levels of anxiety-like behavior while reducing passive stress coping in both strains. Our results highlight the importance of an individual's phenotype to their response

[§]Corresponding author at: Integrated Life Sciences Building (ILSB), 1981 Kraft Drive, Blacksburg, VA, meglover@vt.edu.

to antibiotics, contributing to the understanding of the complex interplay between gut microbes, immune function, and an individual's emotional phenotype.

Keywords

microbiome; anxiety; emotionality; depression; antibiotics; cytokines

Introduction

The microbiome plays an essential role in maintaining gastrointestinal health, but also broadly impacts brain function and behavior via bidirectional communication between the brain and gut microbe communities (Sylvia and Demas 2018; Winter et al. 2018). Gastrointestinal disorders like Irritable Bowel Syndrome are highly comorbid with emotional disorders (Rieder et al. 2017; Mikocka-Walus et al. 2016), and changes in intestinal microbial communities have been linked to a variety of neurological and psychiatric disorders including Alzheimer's disease, Parkinson's disease, autism, anxiety disorders, and depression (Dinan and Cryan 2012; Valles-Colomer et al. 2019). Furthermore, factors that alter the microbiome composition (e.g., antibiotics, obesity, stress) have been shown to induce long-term microbiome dysbiosis and influence psychiatric symptoms (Capuco et al. 2020). For instance, stress alters the microbiome in rodent models (Provensi et al. 2019; Burokas et al. 2017; Werbner et al. 2019). Elevated cortisol can lead to increased gut permeability (Collins and Bercik 2009; Forsythe, Bienenstock, and Kunze 2014; Forsythe et al. 2010; Stilling, Dinan, and Cryan 2014), resulting in translocation of endotoxins and short chain fatty acids produced by bacteria and an increase in pro-inflammatory cytokines (Kiecolt-Glaser et al. 2018; Erny et al. 2015). Several case studies suggest a link between antibiotic treatment and episodes of mania or psychosis in adult patients with existing psychiatric conditions (Walrave, Mohammad, and Ploeger 2016; Saidinejad, Ewald, and Shannon 2005; Stuhec 2014). A population-based study found increased depression and anxiety symptomology in adult patients following antibiotic treatment (Lurie et al. 2015), and another study reported increased risk of mental disorders in children and adolescents that had been treated with antibiotics (Kohler-Forsberg et al. 2018). In both studies, other anti-infective drugs including antivirals and antiparasitics were not associated with increased risk for psychiatric disorders (Kohler-Forsberg et al. 2018; Lurie et al. 2015). Together these findings suggest that: *1)* perturbations of the fecal microbiome may contribute to the biology of emotional disorders; and *2)* preexisting differences in temperament or psychiatric risk may be associated with microbiome variability. There remains a need to better understand how the gut microbiome influences the brain and behavior since it may ultimately pave the way for developing novel therapeutic interventions.

Experimental animals offer a useful tool to study how changes in gut microbiota influence brain function (Bravo et al. 2011; Burokas et al. 2017; Zhang et al. 2017). Mice raised in germ-free conditions lack a microbiome as well as reduced anxiety-like behavior (Clarke et al. 2013; Diaz Heijtz et al. 2011; Neufeld et al. 2011) and deficient cognitive function in social recognition, cued free conditioning, and novel object recognition (Davidson et al.

2018) compared to normally-housed mice of the same strain. Antibiotic treatment induces broad gut microbiome dysbiosis and influences rodent anxiety- and depression-like behaviors, although the effects vary across studies, with some reports showing reduced anxiety-like behavior following antibiotic treatment (Leclercq et al. 2017; Lieve Desbonnet et al. 2015) and others finding increased anxiety-like and/or depression-like behaviors (T. Wang et al. 2015; Ceylani et al. 2018; Hoban et al. 2016). Developmental changes in the microbiome (i.e., through early-life antibiotic treatment or stress exposure) also potently affect emotional behavior. Intestinal microbe communities change dramatically throughout the lifespan, with developmental shifts in microbe communities closely aligning with developmental changes in emotion-regulating brain regions like the amygdala (Scott et al. 2017; O'Toole and Jeffery 2015). Early-life antibiotic exposure in mice leads to reduced anxiety-like behavior and increased social aggression in adulthood (Leclercq et al. 2017; Lieve Desbonnet et al. 2015). Stress exposure in early life is well-known to trigger increased anxiety- and depression-like behaviors in later life (Heim and Binder 2012), and studies in non-human primates (Bailey and Coe 1999) and rodents (De Palma et al. 2015; Moloney et al. 2015; El Aidy et al. 2017; Moya-Perez et al. 2017; McVey Neufeld et al. 2017) suggest these effects are driven, in part, through changes in the gut microbiome. Interestingly, early-life stress-induced anxiety-like behavior was ameliorated by treatment with the probiotic *Bifidobacterium* (Moya-Perez et al. 2017) or a combination of probiotic *Lactobacillus* and prebiotics (McVey Neufeld et al. 2017). Overall, these animal model data are broadly consistent with human findings, lending additional support to the notion that changes in the intestinal microbiome influence cognition and emotional behavior (Sylvia and Demas 2018; Winter et al. 2018; Capuco et al. 2020).

As noted above, a range of environmental factors including antibiotic treatment and stress exposure alter intestinal microbiome, brain function, and behavior (Cowan et al. 2018); however, inconsistent results across studies raise questions regarding why individuals exhibit variable responses to these stimuli. For instance – do inborn differences in temperament influence how an individual reacts to antibiotic treatment (or stress)? Further, what mechanisms potentially drive an individual's behavioral response following antibiotic treatment? To begin to address such questions, the current study utilized a rat model of individual differences in temperament where rats were selectively bred for high versus low behavioral response to novelty. Rats bred for high behavioral response to novelty (High Responders, HRs) display vigorous novelty exploration as well as impulsivity, aggression, and proclivity to addictive behaviors compared to low novelty responder (LR) rats that show high levels of behavioral inhibition, spontaneous anxiety-like behavior, passive stress coping and vulnerability to chronic stress (Stead et al. 2006; Stedenfeld et al. 2011; Garcia-Fuster et al. 2012; Glover et al. 2015; Flagel et al. 2014). To date, it is unknown if HR/LR rats have distinct gut microbiomes or if such differences may contribute to their disparate behavioral phenotypes. Thus, the present study examined microbiota profiles in HR/LR rats. We hypothesized that some aspects of the HR/LR neurobehavioral phenotypes may be driven, at least in part, by microbiome differences and that altering their microbiomes could shift (i.e. normalize) aspects of their behavior.

Our investigation began by treating HR/LR rats with a 24-day course of oral antibiotics to test whether depleting the fecal microbiome would eliminate HR/LR behavior differences. A

second experiment used next-generation sequencing to examine the intestinal microbiomes of adult male HR versus LR rats. Lastly, given known connections between microbiome dysbiosis, immune system factors, and permeability of the intestines and blood-brain barrier, our final experiment assessed potential HR/LR differences in immune system markers and blood-brain barrier permeability, as these represent possible mechanisms whereby the microbiome could impinge on behavior.

Experimental Procedures

All experiments were approved by the local Institutional Animal Care and Use Committee and conducted in accordance with National Institutes of Health guidelines on animal care and experimentation.

Animals

Adult male HR/LR rats were obtained from the 8th generation of our in-house colony, which was described in a recent publication from our group (C R McCoy et al. 2016). Rats were pair-housed with either two HR rats or two LR rats per cage (*i.e.* no HR/LR mixed housing). The housing and testing facilities were maintained at 21–23°C, 50-55% humidity, and the housing room had a 12:12 light-dark cycle (6AM-6PM). Food and water were available *ad libitum*. All animals were between 60-65 days old by experimental day one.

HR/LR Antibiotic Treatment

Antibiotic Treatment—Pair housed HR/LR male rats (n=16/phenotype) were assigned to either antibiotic or control groups by cage. Antibiotic-treated animals received an antibiotic cocktail of ampicillin (0.5g/L), neomycin (1.0g/L), and vancomycin (0.5g/L) dissolved in drinking water starting two weeks prior to embarking on a behavioral test battery. Antibiotic treatment then continued throughout the test period.

Behavioral Testing—Behavioral tests were recorded using a computerized analysis program (Ethovision XT 8.0, Noldus, Wageningen, The Netherlands) and conducted under dim light (30 lux) between 8:00-13:00. Animals were tested over a one-week period with one test conducted per day, beginning with the Open Field Test (OFT) followed by the Elevated Plus Maze (EPM), Defensive Burying test, and Forced Swim Test (FST).

Open Field Test: The OFT was conducted in a 100×100×50 cm black Plexiglas box with a black floor as described (Cohen et al. 2015). At the beginning of the test, a rat was placed in a corner of the box and 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, sides, and corners of the apparatus were quantified. The periphery was defined by a 20 cm zone around the edge of the OF arena that was further subsided into mutually exclusive side (20 × 60 cm) and corner (20 × 20 cm) zones.

Elevated Plus Maze: The EPM test was conducted as described (Cohen et al. 2015) in a black Plexiglas EPM consisting of four elevated arms (70 cm from the floor, 45 cm long, 12 cm wide) arranged in a cross. Two opposite arms are enclosed by 45-cm-high walls (lighting

3–5 lux), and the remaining arms are open (lighting 30 lux). To start the test, rats were individually placed in a central square platform facing the same closed arm at the intersection of the open and closed arms provided access to all arms. The latency to enter the open arms, the amount of time spent in the open arms, closed arms, and center square, and the total distance traveled over the course of the 5-min test were recorded. An animal was considered to be in the open arm when the rat's body fully crossed out of the center square onto an open arm platform.

Defensive Burying Test: The defensive burying test was conducted as previously described (Cohen, Ata, et al. 2017). Rats underwent two daily 15-min habituation trials in a Plexiglas chamber (45cm × 45cm × 60cm, Noldus, Wageningen, Netherlands) filled with 3 inches of clean bedding. 24-h after the final habituation, rats returned to the chamber, which contained an electric probe. When rats interacted with the active electric probe, they received a single 4.0mA shock. Behavior was observed for 15-min following the shock. A blinded experimenter measured time spent immobile (reactive coping); and burying, defined as the rat actively pushing or throwing bedding in the direction of the probe with its front paws or head (proactive coping). The arena was divided into front, middle, and back thirds (15cm × 45cm) and time spent near the probe (front) was measured.

Forced Swim Test: Porsolt's FST was performed as we previously described (Nam et al. 2014; Glover et al. 2015) with 30-cm deep 25°C water in Plexiglas containers (45 cm high x 20 cm diameter). On FST day 1, rats were placed (one per 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 was swimming in clean water. Rats were videotaped during both test days and immobility and swimming were analyzed and used to generate a behavioral z-score for the FST (see analysis description below for further details).

Tissue/Sample Collection and Molecular Analyses

Fecal Samples—Fecal samples were collected prior to the start of antibiotic treatment and on day 23 of antibiotic treatment. Following collection, samples were stored at −80°C until further processing. Gastrointestinal transit time was determined as previously described (Li et al 2011, J. Neuro Gastrointestinal motility). Briefly, a solution of carmine red (6%, Sigma-Aldrich), which cannot be absorbed from the lumen of the gut, suspended in drinking water, was administered via gastric gavage (0.5mL). After gavage, fecal pellets were monitored for the presence of carmine red. The time between gavage and the presence of the dye in a fecal pellet was recorded.

Blood, brain and organ samples—One day (24 h) following the last behavioral test (the FST), a subset of the animals (n=8 per phenotype/treatment) were sacrificed by rapid decapitation, brains were removed, flash frozen, and stored at −80°C until further processing. Trunk blood was collected in 6 mL K_2 EDTA tubes (Fisher). Plasma was separated from whole blood by spinning at 5000 rpm for 20 min at 4°C and stored at −80°C until further processing. To examine potential physical differences in the gastrointestinal tracts of HR/LR rats (either at baseline or following antibiotic treatment). Colon length,

cecum mass, and spleen mass were recorded for each animal. Brains were cryostat sectioned at 300 μm, and collected onto slides. Amygdala and ventral hippocampus were later dissected for RNA extraction and downstream RT-qPCR. Plasma samples were used to determine cytokine levels.

Microbiome Sequencing and Analysis

DNA was isolated from fecal samples with a sample from one rat per cage analyzed to avoid confounding by cage effects. (Because rats are coprophagic, cage-mates commonly exhibit similar microbiomes(Malik et al. 2016; Caruso et al. 2019).) DNA was isolated using Quick-DNA™ Fecal/Soil isolation Kit (Zymo Research). The 16S rRNA V4 region gene was amplified by PCR using previously described primers and the amplicon purified and sequenced using the MiSeq platform (Kumar et al. 2014). Raw FASTQ files were analyzed using DADA2 pipeline of Nephele v2 (OCICB 2018). Reads were quality filtered (maximum expected error of 5 and 0 allowed mismatches between the overlap of merged reads) and grouped into Operational Taxonomic Units (OTUs) using DADA2 (Callahan et al. 2016). This approach is a way to classify a single “bacterial group” that may represent a strain or species of bacteria. A sequencing depth of 72,428 reads per sample was achieved, with an average of 57,415 (79.3%) of merged reads per sample. From this, 810 OTUs were identified. OTU data was not rarified, however OTUs that were present in fewer than 4 of the 24 samples were filtered out prior to all analyses. Differences in OTU abundance between HR/LR, conventionally housed animals (received normal drinking water) or antibiotic-treated animals, and interaction effects were determined using ALDEx2 package in R and Benjamini-Hochberg corrected p-value of <0.05 as the threshold for significance. The ALDEx2 package uses a compositional data analysis that accounts for the proportional nature of RNA-seq datasets, this approach has been shown to be robust for the detection of differentially expressed genes/features and to reduce the number of false positives (Fernandes et al. 2013; 2014). Principal Component Analysis using Aitchison distance was performed on relative abundance OTU tables (Aitchison 1982). Simpson’s Diversity and Shannon Diversity were chosen as measures of species evenness. Principle component analysis and diversity plots were generated using Phyloseq (McMurdie and Holmes 2014). Permanova analysis was used to statically determine group differences in microbiome composition using the adonis2() function from the “vegan’ package in RStudio (Version 1.0.153, R. RStudio, Inc., Boston, MA).

Plasma Cytokine and Hormone Assay

To examine potential immune system differences in HR/LR control and antibiotic-treated rats, we used a MILLIPLEX® MAP Rat Cytokine/Chemokine Magnetic Bead Panel (RECYTMAG65K27PMX, EMD Millipore), which measures levels of 27 cytokines and chemokines in rat plasma. Plasma samples were stored at −80C, then thawed to room temperature, vortexed and centrifuged at 1000g for 10 minutes prior to use. Samples were processed according to the manufacturer’s recommended protocol, then run on a Luminex MAGPIX and quantified using MILLIPLEX® Analyst 5.1 software. The cytokines/chemokines measured included the following: G-CSF, Eotaxin, GM-CSF, IL-1α, Leptin, MIP-1α, IL-4, IL-1β, IL-2, IL-6, EGF, IL-13, IL-10, IL-12p70, IFN, IL-5, IL-17A, IL-18, MCP-1, IP-10, KC, VEGF, Fractalkine, LIX, MIP-2, TNFα, and RANTES.

Blood Brain Barrier Permeability

Past studies suggest that blood brain barrier (BBB) permeability represents a key mechanism whereby the gut microbiome impacts brain function(Pfau, Ménard, and Russo 2018). To begin to assess BBB permeability in HR/LR rats (with and without antibiotic exposure), we processed brain tissue with Evans Blue, a commonly used (albeit imperfect) marker of brain barrier integrity (Saunders et al. 2015) in a separate cohort of control and antibiotic-treated HR/LR rats. Experimental animals (n=6 per phenotype/treatment) were deeply anesthetized with sodium pentobarbital (150 mg/kg i.p.) and then transcardially perfused with ~100 ml of physiological saline followed by ~300 ml of 1% Evans Blue dissolved in 4% paraformaldehyde. Brains were extracted, post-fixed overnight, cryoprotected in 20% sucrose, and stored at −80°C. Briefly, brains were sectioned coronally on a freezing microtome at a thickness of 40 μm. Tissue was examined using an Olympus BX-UCB microscope (http://www.olympusamerica.com/) equipped with a motorized stage and a cooled mono CCD camera (Orca R2; Hamamatsu, http://hamamatsucameras.com/). Serial sections of basolateral amygdala and ventral hippocampus were imaged at high magnitude (20X, 0.2 s exposure time, 4dB gain) to examine fluorescence of the entire region. Relative optical densities were determined using ImageJ (NIH). Mean relative optical density of the entire region was found by tracing the entire region and measuring optical density with ImageJ. Three to six vessels per region were averaged together for each animal.

Assessing expression of blood brain barrier-related genes

Another approach for assessing BBB integrity is to examine expression of tight junction markers that comprise the barrier. Thus, we conducted quantitative Real Time PCR (qRT-PCR) using a Quantstudio 6 (Applied Biosystems, Grand Island, NY, USA) with TaqMan detection chemistry as previously described (Cohen et al. 2015; Glover et al. 2018) to determine baseline HR/LR gene expression differences in multiple tight junction markers. We examined the expression of the following genes: *Cldn1* (Rn00581740_m1), *Cldn3* (Rn00581751_s1), *Cldn5* (Rn01753146_s1), and the endogenous control gene *Gapdh* (Rn01775763_g1). Relative fold changes between antibiotic-treated versus control HR/LR groups were compared for a given gene at a particular time point were calculated using the ΔΔCT method.

Data analysis

Data analyses for the next-generation sequencing and prior transcriptome study are described above. Data from the qRT-PCR and BBB permeability studies were analyzed using GraphPad Prism Software (Version 6.0, GraphPad, La Jolla California USA). Data were verified to be normally distributed and then analyzed using two-way ANOVAs with phenotype and antibiotic treatment as independent variables. Tukey multiple test correction was used when appropriate. Behavioral measures from the OFT, EPM, defensive burying test, and FST were analyzed using two-way ANOVAs with phenotype and antibiotic treatment as independent variables. Tukey multiple test correction was used when appropriate. Significance was set at $p < 0.05$, and results are presented as mean ± SEM.

In addition to standard analysis of behavioral measures between experimental groups (e.g., time spent in the center of the OF; latency to enter the OF), we also used Z normalization

values, which are dimensionless tools that compare experimental group means across behavioral measures both within and between behavioral tests (Guilloux et al. 2011; Glover et al. 2018). This type of analysis offers an estimate of an animal's gestalt emotionality since it takes into account behavioral responses across a range of tests of emotional behavior. The Z value represents how many standard deviations (σ) a value (X) is above or below the group average (μ) and is represented by the equation: $Z = X - \mu/\sigma$ where μ and σ are the mean and standard deviation, respectively, of the control group. For each behavioral test, all groups were normalized to the control HR cohort. In the anxiety- and coping style measures, a positive change from baseline indicates increased anxiety-like behavior (*e.g.*, time spent in anxiogenic region, novelty exploration) or passive coping (*e.g,* FST immobility, defensive burying immobility). When correlating behavioral measures with cytokine levels, z-scores were used in place of the raw behavioral data. Z-scores were also used in the analysis of BBB permeability of Evan's Blue dye.

Results

Effects of antibiotic treatment on gastrointestinal system.

The experimental timeline from the start of antibiotic treatment through the end of behavioral testing and sample collection is depicted in Figure 1A. There were no differences in group weight (Fig. 1B, see Table 1 for ANOVA results). There was no significant effect of HR/LR phenotype ($F(1,12)=0.01879$, $p=0.8932$), treatment ($F(1,12)=4.354$, $p=0.0589$), and phenotype x treatment interaction ($F(1,12)=0.1252$, $p=0.7296$) in water consumption by weight (Fig. 1C). Gastrointestinal transit was similar in HR versus LR rats (no effect of phenotype), but was significantly increased by antibiotic treatment ($F(1,28)=31.84$, $p<0.0001$; Fig. 1D). Colon length and spleen mass was similar across groups (no effect of phenotype or antibiotic treatment; Fig. 1E-F), although antibiotic treatment lead to a dramatic increase in cecum size in both HR and LR rats (main effect of antibiotic treatment, $F(1,28)=99.67$, $p<0.0001$; Fig.1G).

Antibiotic treatment exacerbates select aspects of the HR/LR behavioral phenotypes

Several prior studies have described how HR/LR rats display distinct behavioral phenotypes in a range of tests of emotional behavior, with LR rats generally displaying high levels of behavioral inhibition, anxiety-like behavior, and passive stress coping relative to HR rats (Stead et al. 2006). Because gut microbiome composition has been shown to influence these types of behaviors (e.g., (Moya-Perez et al. 2017; McVey Neufeld et al. 2017; Hoban et al. 2016)), we hypothesized that HR/LR microbiome differences may contribute to their disparate behavioral phenotypes. Thus, we treated HR/LR rats with a course of antibiotics to test whether disrupting the HR/LR microbiomes would alter aspects of their behavior in tests of anxiety (OFT, EPM) and stress coping (defensive burying, FST).

Behavioral data are presented as normalized z-scores to allow for analysis of related behavioral measures in a combinatorial fashion. This normalization procedure reduces the intrinsic variability of single tests by combining similar measures within and between tests to produce an emotionality z-score (Guilloux et al. 2011; Glover et al. 2018). For each test, all behavioral measures were converted so that increased z-scores reflect increases in

anxiety-like (center time in OFT, time in open arms of EPM, and locomotor activity in both; depicted in Fig. 2A-C) and passive stress coping behavior (defensive burying, FST immobility (depicted in Fig. 2D-F). Raw behavioral data (mean ± SEM) included to generate the z-scores is in Table 2.

In the OFT, LR rats displayed typically high levels of anxiety-like behavior relative to HRs (main effect of phenotype, $F(1,28)=66.48$, $p<0.0001$; Fig. 2A). Although there was no overall effect of antibiotic treatment, there was a phenotype x antibiotic treatment interaction ($F(1,28)=12.53$, $p=0.0014$). Post hoc analysis revealed increased anxiety-like behavior in antibiotic-treated LRs versus LR controls ($p=0.0098$). In the EPM, the LRs again (regardless of antibiotic treatment group) showed higher anxiety-like behavior compared to HRs (effect of phenotype, $F(1,28)=24.17$, $p<0.0001$; Fig. 2B). There was no effect of antibiotic treatment and no phenotype x antibiotic treatment interaction on EPM score. When considering overall anxiety score across both the OFT and EPM, there was a main effect of phenotype ($F(1,24)=57.04$, $p<0.0001$) and a phenotype x antibiotic treatment interaction ($F(1,24)=8.362$, $p=0.0073$). LR rats generally showed higher levels of anxiety relative to HRs, with LR controls showing higher anxiety compared to HR controls ($p=0.0027$) and antibiotic-treated LRs also showing higher levels of anxiety relative to antibiotic-treated HRs ($p<0.0001$). Interestingly, antibiotic treatment appeared to further exacerbate LRs' high anxiety levels as antibiotic-treated LRs displayed significantly higher anxiety compared to the LR control group ($p=0.0215$) groups (Fig. 2C).

The Defensive Burying test and FST both challenge an animal with a stressful situation that can be dealt with in one of two ways: *a)* an active stress coping approach (i.e., burying the shock probe in the Defensive Burying test; swimming, climbing, or escape attempts in the FST); or *b)* a passive stress coping approach (i.e. freezing or avoiding the shock probe in the Defensive Burying test; immobility in the FST). In the Defensive Burying test, LR rats showed high levels of passive coping relative to HRs (effect of phenotype, $F(1,24)=24.01$, $p<0.0001$; Fig. 2D). There was no effect of antibiotic treatment, but there was a significant phenotype x treatment interaction ($F(1,24)=8.486$, $p=0.0076$). Post hoc analysis indicated that antibiotic-treated HRs showed less passive coping compared to HR controls ($p=0.0162$) and antibiotic-treated LRs ($p<0.0001$) (Fig. 2D). In the FST, LR rats showed high levels of passive coping relative to HRs (effect of phenotype, $F(1,28)=15.07$, $p=0.0006$; Fig. 2E). There was also a significant effect of antibiotic treatment ($F(1,28)=11.70$, $p=0.0019$), but no phenotype x antibiotic treatment interaction. Antibiotic treatment lead to reduced passive coping behavior in both HR and LR rats compared to controls.

When considering overall stress coping score (combining measures across the FST and Defensive Burying test), there were main effects of phenotype ($F(1,24)=33.40$, $p<0.0001$), antibiotic treatment ($F(1,24)=9.286$, $p=0.0052$), and a phenotype x treatment interaction ($F(1,24)=5.650$, $p=0.0251$). LR rats overall displayed higher levels of passive coping relative to HRs (Fig. 2F). Antibiotic treatment increased active stress coping HR rats ($p=0.0010$ for post-hoc comparison of HR control versus antibiotic-treated HR; Fig. 2F), while there was no effect on the LRs' coping style.

Adult male HR/LR rats exhibit limited microbiome differences.

Using a next generation sequencing approach, we examined the fecal microbial content of HR and LR rats at baseline and following antibiotic treatment. Our first phase of analysis used principle components of analysis, a data reduction technique that apportions the major components of variance within a dataset into a limited number of dimensions. The greatest variability between samples was attributed to antibiotic treatment (27.7%), with no notable effect of HR/LR phenotype (Fig. 3A). This analysis also revealed that antibiotic treatment drastically reduced microbiome heterogeneity in both HR and LR rats (Fig. 3A). Permanova analysis found a main effect of treatment ($p=0.001$) but no significant effect of strain ($p=0.274$) and no strain x treatment interaction ($p=0.436$) (full results reported in Table 1). We examined microbiota community diversity using Simpson and Shannon alpha diversity indices where higher index values indicate greater density of unique OTUs. This analysis revealed a main effect of antibiotic treatment in both Simpson ($F(1,20)=420.1$, $p<0.0001$), and Shannon ($F(1,20)=335.0$, $p<0.0001$) alpha diversity indices (Fig. 3B-C) with no effect of HR/LR phenotype. Sunburst charts (Fig. 3D) illustrate the phylogenetic tree of the microbiome in HR controls, LR controls, antibiotic-treated HRs, and antibiotic-treated LRs. Charts are organized such that the innermost ring represents the phyla (shown in key), ending at the species level in the outermost ring. A list of the OTUs used to create these plots can be found in Supplemental Table 1.

The heatmap in Figure 4A depicts relative abundances of multiple taxa in antibiotic-treated and control HR/LR rats, and it also illustrates the dramatic decrease in microbial heterogeneity following antibiotic treatment. Volcano plots of the ALDEx2 analysis are used here to demonstrate *a)* zero OTUs that significantly differed between HR/LR control rats (Fig. 4B); *b)* 115 OTUs that differed between control and antibiotic-treated rats (Fig. 4C); *c)* 44 OTUs that differed between control and antibiotic-treated HR rats (Fig. 4D); and *d)* 68 OTUs that differed between control and antibiotic-treated LR rats (Fig. 4E). A list of OTUs significantly altered by antibiotic treatment are included in Supplemental Table 2. The results of a correlation analysis of those OTUs significantly altered in LRs or HRs, but not both, with the behavioral measures is included in Supplemental Table 3.

Evaluating potential HR/LR differences in immune system markers

Given the known relationships between gut microbiota, immune system factors, brain function and emotional behavior, we wanted to examine possible HR/LR differences in immune markers that could contribute to HR/LR phenotypic differences. Thus, at the conclusion of the behavioral test battery, we collected trunk blood from HR/LR rats and the plasma was analyzed using a multiplex cytokine/chemokine panel. This assay allowed us to compare a range of inflammatory and immune markers across experimental groups (control and antibiotic-treated HR/LR samples).

Two factors on the multiplex assay showed differences between experimental groups – leptin and IL-1β. Leptin, a hormone long known to regulate appetite, also plays a role in modulating the emotional response to stress and promotes an inflammatory response (Van Doorn et al. 2017; McGregor and Harvey 2017; La Cava 2017). We found that antibiotic treatment led to reduced leptin levels (main effect of antibiotic treatment, $F(1,26)=27.02$,

p<0.0001; Fig. 5A). Although there was no significant effect of phenotype on leptin levels, there was a phenotype x treatment interaction (F(1,26)=5.213, p=0.0308). Post hoc analysis showed that *a)* control LRs had higher leptin levels compared to control HRs (p=0.0223); and *b)* antibiotic treatment significantly reduced leptin in HRs (p=0.0425) and LRs (p<0.0001) relative to their respective control groups. Linear regression demonstrated a positive correlation of leptin with passive coping (R^2=0.1850, p=0.0177; Fig. 5B), but there was no significant correlation between leptin and anxiety-related measures.

IL-1β is a pro-inflammatory cytokine that can be produced and released by a wide variety of cell types and is a part of the innate immune response (Lopez-Castejon and Brough 2011). We found no main effect of HR/LR phenotype (F(1,25)=1.426, p=0.2436) or antibiotic treatment (F(1,25)=0.3231, p=0.5748) on IL-1β levels; there was a significant phenotype x treatment interaction (F(1,25)=4.366, p=0.0470). Post hoc analysis showed that antibiotic treatment specifically reduced IL-1β levels in HRs (p=0.0264). Antibiotic treatment eliminated baseline HR-LR IL-1β differences (Fig. 5C).

Blood brain barrier permeability & tight junction marker expression.

Because BBB permeability represents a key mechanism whereby the gut microbiome impacts brain function (Pfau, Ménard, and Russo 2018; Braniste et al. 2014; Martin et al. 2018), and considering the potential influence of leptin on BBB permeability (Geng et al. 2018; Stranahan et al. 2016; Corem et al. 2019), we next sought to determine if antibiotic treatment differentially impacted BBB permeability in HR/LR rats. We hypothesized that antibiotic exposure would lead to increased BBB permeability in one or both strains due to the observation of reduced serum leptin in antibiotic-treated HR/LR animals. We assessed BBB integrity in two ways, first using Evans Blue staining and secondly by measuring expression of tight junction proteins *Cldn1, Cldn3*, and *Cldn5*, which are key components of the BBB (Obermeier, Daneman, and Ransohoff 2013; Keaney and Campbell 2015). These studies focused on two brain regions known to regulate emotional behavior and known to differ in the HR/LR brain – the basolateral amygdala and ventral hippocampus.

The Evans Blue study in basolateral amygdala revealed increased BBB permeability in antibiotic-treated rats (effect of antibiotic treatment, F(1,19)=6.339, p=0.0209; Fig. 6A), with no apparent HR/LR phenotype difference and no phenotype x antibiotic interaction. Results of the qPCR studies in the amygdala showed that antibiotic treatment lead to decreased levels of *Cldn1* (main effect of antibiotic treatment, F(1,22)=5.413, p=0.0296), but it did not affect *Cldn3* or *Cldn5* (Fig. 6B). There were no effects of phenotype and no phenotype x antibiotic treatment interactions for these tight junction genes.

In the ventral hippocampus, the Evans Blue study revealed a main effect of phenotype (F(1,19)=5.326, p=0.0324) with evidence of increased BBB permeability in HR versus LR rats (Fig. 6C). There was no effect of antibiotic treatment, and no phenotype by antibiotic interaction on BBB, in the ventral hippocampus. The qPCR experiments to evaluate tight junction genes in the ventral hippocampus showed higher levels of *Cldn3* in LRs versus HRs (effect of phenotype F(1,24)=12.47, p=0.0017; Fig. 6D). There were no effects of antibiotic treatment and no antibiotic x phenotype interaction for *Cldn3*. Likewise, there were no

effects of phenotype, antibiotic treatment or phenotype x antibiotic interaction on *Cldn1* or *Cldn5* expression.

Discussion

Emotional behavior and well-being relies on a complex interplay of neural, endocrine, and physiological systems, and there is a growing appreciation of a role for intestinal microbiota in regulating these processes (Dinan and Cryan 2012). Microbiome abnormalities have been reported in a number of neuropsychiatric disorders, and work in experimental animals demonstrates that manipulating the microbe composition (*e.g.*, via raising rodents in germ-free conditions; treating animals with antibiotics, prebiotics or probiotics) can modulate many behavioral domains, including anxiety- and depression-related behaviors (Hoban et al. 2016; Burokas et al. 2017). The present study aimed to: *1)* characterize microbiome profiles in selectively bred HR/LR rats that exhibit distinct emotional behavior phenotypes; *2)* test whether antibiotic-induced dysbiosis alters HR/LR behavior differences; and *3)* examine possible biological mechanisms whereby the gut microbiome may influence HR/LR neurobiology and behavior. Overall microbiome composition was similar in baseline HR/LR rats. A four-week course of antibiotics had largely similar effects on the HR and LR microbiomes. Most of the taxa significantly altered by antibiotic treatment belong to the Clostridiales and Bacteroidales orders, which is not surprising given those orders made up the majority of the microbiome population (based on number of OTUs). We found that antibiotic treatment altered HR/LR behavior, although it did so in an unexpected manner. Rather than attenuating HR/LR behavior differences as we predicted, antibiotic treatment exacerbated some HR/LR behavioral differences. We found that antibiotic-treated LRs showed even higher than usual levels of anxiety-like behavior while antibiotic-treated rats of both strains displayed greater amounts of active stress coping (i.e., in the FST and defensive burying test) than usual. We also found novel correlations between some of our behavior behavioral measures and circulating levels of leptin, as well as region and strain-specific effects on blood-brain barrier permeability. Overall, these findings point to novel factors that participate in the complex interplay between the gut microbiome and an individual's emotional phenotype.

Effects of antibiotics on fecal microbiome and behavior in HR/LR rats

Treating HR and LR rats for 24 days with vancomycin, ampicillin, and neomycin led to reduced microbiome richness and diversity. Two common measures of microbiome diversity are the Shannon and Simpson indices, which measure microbial species richness (number of OTUs present in a sample) and evenness (the distribution in OTU abundance) (Kim et al. 2017; Lemos et al. 2011; SIMPSON 1949). The Shannon index puts greater weight on species richness over evenness while the Simpson index more heavily weighs evenness over richness. More specifically, antibiotic treatment led to reduced richness of the dominant Bacteroidetes and Firmicutes phyla, and proliferation of Proteobacteria. In LRs, antibiotic treatment also led to proliferation of Firmicutes (Clostridiales order) that we did not see in HRs.

Vancomycin, routinely used to treat *Clostridium difficile (C. difficile.)* (Banawas 2018), depletes Firmicutes and Bacteroidetes in the intestines and allows for proteobacteria proliferation (Isaac et al. 2017). More specifically, studies in mice (Lewis et al. 2015) and humans (Louie et al. 2012; Isaac et al. 2017), and the data reported here in rats, all demonstrate vancomycin-mediated, and host-species independent, proliferation of *Klebsiella* and *Escherichia/Shigella*. Notably, this proliferation occurred in both rat lines studied here. Interestingly, several Firmicutes of the order Clostridiales proliferated in LRs, but not HRs, following antibiotic treatment. While vancomycin was developed for the treatment of *C. difficile*, ampicillin is implicated in causing *C. difficile* infections (Ofosu 2016).

Antibiotic treatment altered a few dozen OTUs of the order of spore-forming bacteria Clostridiales, including increased abundance of two OTUs in the Clostridiales order in LRs but not HRs. Unfortunately, species level info on these taxa was not obtained; however, the proliferation of Clostridiales following broad-spectrum antibiotic treatment has been observed clinically. *C. difficile.* proliferation is normally inhibited in the presence of other gut microbes; however, when competition is low, *C. difficile* may proliferate (Theriot and Young 2015; Rodriguez et al. 2016). The abundance of Clostridium genera has been negatively correlated to the severity depressive symptoms, while fecal butyrate (a product of *Cl. butyricum*) concentration was negatively correlated with both depression and anxiety symptoms (Borgo et al. 2017). *C. butyricum,* was recently tested as an adjunct to selective serotonin reuptake inhibitor (SSRI) therapy for the treatment of depression and was found to produce greater reductions in depressive symptoms than SSRI treatment alone (Miyaoka et al. 2018). In C57BL/6 mice, social defeat reduced the abundance of Clostridial species (Qu et al. 2017). In another study in C57BL/6 mice, supplementation with *C. butyricum* attenuated depressive-like behavior induced by chronic unpredictable mild stress and increased hippocampal serotonin and BDNF concentrations (Sun et al. 2018). It is possible that either depletion of taxa or the proliferation of specific taxa may drive the disparate effects of antibiotic treatment on HR/LR rats.

As noted above, a surprising aspect of our antibiotic treatment study was the fact that antibiotics exacerbated select aspects of the HR and LR phenotypes. Previous work in humans (Kleiman et al. 2015; Lurie et al. 2015) and antibiotic-treated rodents (Hoban et al. 2016; L Desbonnet et al. 2014; Sudo et al. 2004; Sampson and Mazmanian 2015) suggested an inverse relationship between gut microbial diversity and depression-like behavior, where diminished microbial diversity was associated with greater anxiety- and/or depression-related behavior. We found that reduced microbial richness and evenness (following antibiotic treatment) is associated with increased anxiety-like behavior in LR rats, but not HRs. Reduced microbial diversity was associated with a shift from passive to active coping (FST and defensive burying test), although this was only apparent in HRs in both tests. Our findings are consistent with a handful of studies using stress-sensitive “LR-like” rodents such as BALB/c mice and Fisher 344 rats that naturally show high levels of anxiety-like behavior that is further exacerbated in germ-free conditions (Nishino et al. 2013; Crumeyrolle-Arias et al. 2014). On the other hand, prior studies using other rodent lines (e.g. NMRI mice, Swiss Webster mice) showed reduced anxiety-like behavior in the germ-free conditions (Diaz Heijtz et al. 2011; Neufeld et al. 2011). When correlating OTUs significantly altered by treatment in LRs but not HRs, we found only one OTU significantly

correlated with anxiety-like behaviors. This was one of the few OTUs that proliferated in the antibiotic treated LRs, suggesting proliferation of this member of the Clostridiales order may drive increased anxiety-like behavior. Together these findings suggest that behavioral responses to antibiotic treatment depend upon an individual's baseline temperamental phenotype and other biological characteristics. For instance, HR/LR rats exhibit differences in a number of neurochemical and neuroendocrine factors (S. Clinton et al. 2008; Kerman et al. 2012; S. M. Clinton et al. 2012; Turner et al. 2011), including leptin, which might play a role in the observed divergent behavioral effects of antibiotic treatment.

Cytokine differences in HR/LR rats at baseline and following antibiotic treatment

Overall, our multiplex assay evaluating several factors related to inflammation and immune function revealed few HR/LR differences. We found altered circulating leptin levels in baseline HR/LR rats (increased in LRs versus HRs), and decreased leptin levels in both strains following antibiotic treatment (Fig. 5A); as well as increased IL-1b at baseline in HRs vs LRs, and an antibiotic-mediated decrease in HRs, but not LRs (Fig. 5C).

IL-1β is a pro-inflammatory cytokine that is a part of the innate immune response (Lopez-Castejon and Brough 2011). This is the first study to report baseline differences in IL-1β in the HR/LR model. Notably, antibiotic treatment reduced IL-1β in HRs to levels measured in LRs. In animal models, there appears to be a complex relationship between, IL-1β, active/passive coping, and the stress response. Passive coping strategies are typically associated with elevated central and peripheral IL-1β (Joana et al. 2016; Wood et al. 2015; Finnell et al. 2017); however, some studies (including the present study) have found elevated IL-1β in animals that adopt active coping strategies (De Miguel et al. 2011). The differences between these reports could be due to the variety of stress protocols implemented or region/tissue analyzed in each. Some stress paradigms can elevate IL-1β in both active and passive coping animals (De Miguel et al. 2011), while others only found either no change (Finnell et al. 2017; Joana et al. 2016; Wood et al. 2015) or, in certain brain regions, even reduced IL-1β in active coping animals (Wood et al. 2015). Together these findings suggest a potential role of IL-1β in mediating stress coping strategies; however, more work needs to be done to parse this relationship.

Leptin is released by adipose tissue and one of its primary functions is to inhibit hunger and stimulate satiety through activation of receptors in the hypothalamus. Leptin has also been shown to influence emotional behavior, stress responsivity, and cognition through activation of receptors in the hippocampus (Van Doorn et al. 2017; McGregor and Harvey 2017; Agusti et al. 2018; Zanini et al. 2017). A prior transcriptome study in the HR versus LR brain revealed altered expression of protein-coding transcripts that regulate leptin receptor expression, including decreased Leptin receptor overlapping transcript-like 1 (*Leprotl1*) in the amygdala of adult LR versus HR males (41). *Leprotl1* is a protein-coding gene that negatively regulates leptin receptor and growth hormone receptor expression (65). Future studies will pursue the role of leptin signaling in the HR/LR brain to determine its role in driving aspects of their divergent behavioral profiles.

The leptin receptor is expressed at various barriers to the circulatory system, and ligand binding has been shown to alter gastrointestinal (Nagpal et al. 2018) and blood-brain

permeability (Stranahan et al. 2016; Corem et al. 2019). Here we found increased serum leptin in LRs versus HRs and antibiotic treatment reduced leptin in both strains. There is prior evidence demonstrating leptin levels are reduced following rapamycin antibiotic treatment; however, we did not see an antibiotic-associated change in weight gain as previously described (Scarpace et al. 2016).

Leptin-deficient rodent models tend to develop hyperphagia-induced obesity and exhibit reduced locomotor activity (Ribeiro et al. 2011). Our study revealed a positive correlation between leptin and passive coping in the FST and found that the highly passive coping LRs had higher leptin levels relative to HRs. These findings stand in contrast with previous reports showing antidepressant-like effects of leptin. Acute systemic injections of leptin have been shown to have an antidepressant-like effect in adult C57BL/6J mice (Liu et al. 2010). Both chronic unpredictable stress and social defeat paradigms have been shown to decrease plasma leptin in Sprague-Dawley rats, and acute intra-hippocampal injections of recombinant rat leptin has an antidepressant-like effect (Lu et al. 2006). Interestingly, systemic leptin injections increased cFos immediate early gene expression in the hippocampus, indicating its role in modulating coping strategies may be through enhanced hippocampal activation (Lu 2007). In the context of the current study, central activation of the leptin receptor may have strain-dependent effects on behavior based on pre-existing differences in this system in HR versus LR rats.

Examination of the blood brain barrier

Our final set of studies aimed to examine integrity of the BBB in HR/LR rats at baseline and following antibiotic treatment. The BBB is the gateway whereby various molecules and nutrients enter the brain, and its structure and function are influenced by several factors, including exposure to stress, immune system activation, hormone receptor binding, and as well as elements of the microbiome (Pearson-Leary et al. 2017; Lehmann et al. 2018; Kealy, Greene, and Campbell 2018). While a myriad of factors may alter BBB permeability, here we will discuss two mechanisms: enterotoxin- and leptin-mediated effects on the blood-brain barrier.

The proliferation of two OTUs in the Enterobacteriaceae family points to one potential mediator of increased BBB permeability we observed. In this study, we observed proliferation of *Escherichia/Shigella* and *Klebsiella* in both HR and LR rats following antibiotic treatment. *Klebsiella oxytoca* enterotoxins can induce intestinal barrier leakage through reduction in claudin expression (Hering et al. 2019). Likewise, *E. coli* enterotoxins have been shown to disrupt tight junctions by triggering redistribution of claudin-1 within the cell (Nassour and Dubreuil 2014) and disrupt the blood-brain barrier (Yang et al. 2019).

A recent study found increased BBB permeability in leptin deficient mice may be a transitory state in the development of diabetes in the model (Corem et al. 2019), which raises the question of whether an antibiotic-induced decrease in leptin trigger a breakdown of the BBB? Reanalysis of previously published transcriptomic data from the amygdala and hippocampus of adult male LR vs HR rats found decreased *leprotl1*, which regulates expression of leptin receptor on endothelial cells in the brain. In our analysis of the BBB of HR/LR rats, we hypothesized that the antibiotic induced reduction in circulating leptin may

alter BBB permeability in the amygdala. Our current investigation focused on the amygdala as well as the ventral hippocampus because these brain regions are known to regulate anxiety-like behavior and stress coping (G.-W. Wang and Cai 2008; Andolina et al. 2013) and because these regions have previously been found to differ in HR/LR rats (Cohen, Jackson, et al. 2017; Chelsea R McCoy et al. 2017; Cohen, Ata, et al. 2017; Cohen et al. 2015; Simmons et al. 2012; Turner et al. 2011).

Decreased expression of tight junction proteins (mainly occludin and claudin-5), as well as diffuse and disorganized tight junctions, imply an increase in permeability of the BBB to inflammatory markers, microbial metabolites, and other peripheral factors (Wiley et al. 2017). In the ventral hippocampus we found HR/LR strain differences, that were unaffected by antibiotic treatment, with HRs exhibiting increased BBB permeability and reduced claudin-3 expression compared to LRs. It is likely the observed differences in BBB permeability are due to some other factor not measured in this study. The increased BBB permeability in the ventral hippocampus of HRs compared to LRs is in line with a recent meta-analysis of HR/LR hippocampal transcriptomic data were the authors found enrichment of endothelial cell transcripts in the hippocampus of LRs vs HRs at P14 and P60 (Birt et al. 2020). Taken together, the reduced expression of endothelial cell transcripts across development, reduced Claudin-3 expression, and increased Evan's Blue leakage into the ventral hippocampus suggests the presence of either an innate genetic difference or a differential response to the early life environment that drives HR/LR differences in endothelial cell function or development.

Braniste et al. demonstrated that germ-free mice had decreased expression of occludin and claudin-5 and that fecal transplant normalized expression of the tight junction proteins (Braniste et al. 2014). Likewise, antibiotic-induced gut dysbiosis has also been shown to down-regulate occludin and claudin-5 in the hippocampus, up-regulate *Tjp1* and occludin in the amygdala, but no differences were noted in the prefrontal cortex or hypothalamus (Frohlich et al. 2016), indicating that systemic antibiotic administration may lead to brain region-specific effects on BBB permeability. Our findings in the basolateral amygdala indicated enhanced BBB permeability following antibiotic exposure, with increased Evan's blue dye leakage in tissue surrounding blood vessels and reduced claudin expression in HR and LR antibiotic-exposed rats. Given the HR/LR differences in circulating leptin and leprotl1 mRNA expression in the adult amygdala, it is possible the observed BBB permeability differences following antibiotic treatment in the basolateral amygdala are driven, in part, by the response to leptin at the BBB. Leptin receptor deficient mouse models exhibit increased BBB leakage, infiltration of macrophages, and pro-inflammatory cytokine production by resident microglia compared to wild type; preventing breakdown of the BBB reversed these effects (i.e. reduced macrophage infiltration and proinflammatory cytokines) in the leptin receptor deficient mice (Stranahan et al. 2016). Prior studies in HR/LR rats have found increased microglia-specific transcripts in the hippocampus LRs vs HRs at postnatal day 14 and in adults (Birt et al. 2020), as well as the amygdala and dorsal raphe in adults (Cohen, Ata, et al. 2017). Future studies will determine the effects of manipulating leptin signaling in the basolateral amygdala on BBB permeability and behavior and seek to determine whether this change mediates central infiltration or expression of inflammatory markers (including IL-1β) or other peripheral factors, such as microbial metabolites.

Technical Considerations.

An important limitation of the present study is that it focused only male HR/LR rats and did not include females. Our previous work with the HR/LR model shows that LR females display high levels of anxiety- and depression-related behaviors akin to their male LR counterparts (Stead et al. 2006; Davis et al. 2008; Cummings et al. 2011). Future studies will investigate potential microbiome, immune system, and BBB markers in HR/LR females. A recent study found antibiotic treatment has a sexually dimorphic effect on microglia transcriptome in mice (Thion et al. 2018), so it would also be interesting to determine whether antibiotic treatment elicits similar or disparate effects on emotional behavior in HR/LR females compared to what was observed in HR/LR males.

In this study, we treated the rats with antibiotics (via drinking water) for 24 days. While this is a longer treatment course compared to some studies (Lewis et al. 2015), other studies have treated animals for 4 to 6 weeks (Kelly et al. 2016; Hoban et al. 2016). We chose a longer course in part to maintain treatment throughout behavioral testing. Lewis et al. demonstrated within 1 day following the cessation of treatment, the microbiome continued to change for up to 3 weeks. Discontinuing treatment prior to behavioral testing may have allowed for further alterations to the microbiome that may have resulted in different behavioral findings. Future studies could parse out the difference in the behavioral effects of antibiotic-induced dysbiosis in the short-term (during treatment) and in the long-term (after cessation, either during or following “recovery” of the microbiome).

Conclusions

In summary, the present studied identified novel effects of antibiotic treatment on gut microbiota, leptin, blood-brain barrier permeability, and rodent emotional behavior. Antibiotic treatment altered emotional behavior in HR/LR rats in a phenotype-dependent manner, increasing the already high anxiety-like behavior of LRs and increasing active coping in HRs. We also demonstrated correlations between leptin and coping behavior. There is ample prior evidence that serum levels of leptin are altered in mood disorders. Whether the observed effects of antibiotic treatment and central leptin activity give rise to mood disorders, or if the observed differences are symptomatic of the disorders, remains unclear; however, future studies should aim to identify if baseline differences in peripheral and/or central leptin activity mediate individual differences in behavioral response to antibiotic treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Antibiotic treatment increased anxiety-like behavior in rats with innately high, but not low, anxiety-like behavior.
- Proliferation of bacteria following antibiotic treatment differed between the rat strains.
- Circulating leptin levels differed between rat strains before treatment, and treatment reduced leptin.
- Antibiotic treatment altered blood-brain barrier permeability in a strain- and region-specific manner.

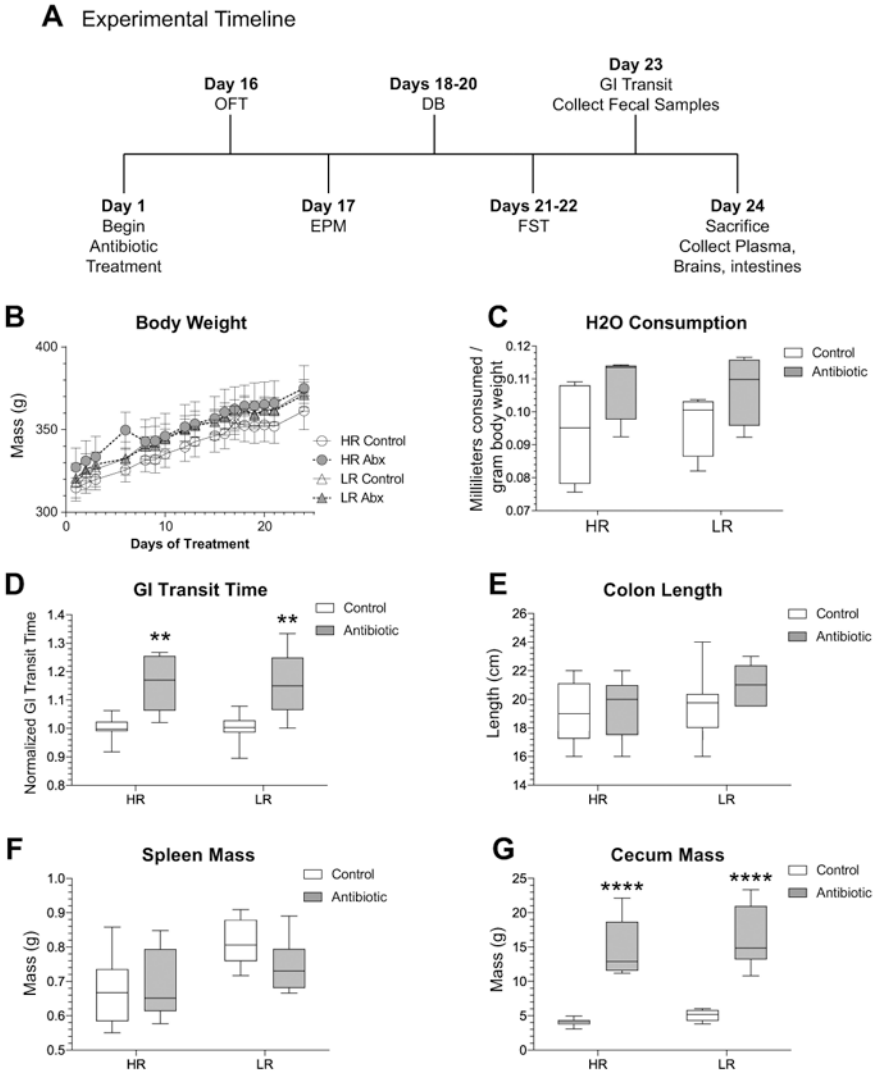

Figure 1: Effects of antibiotic treatment on gastrointestinal system of HR/LR rats.

Experimental timeline of antibiotic treatment, behavioral testing, and sample collection (**A**). Antibiotic treatment had no effect on HR/LR weight gain or water consumption (**B-C**). Antibiotic treatment did increase gastrointestinal transit time of orally administered Carmine Red dye (**D**). While colon length and spleen mass were not affected, antibiotic treatment did significantly increase cecum mass in both HRs and LRs (**E-G**). ** denotes p<0.01, **** p<0.0001.

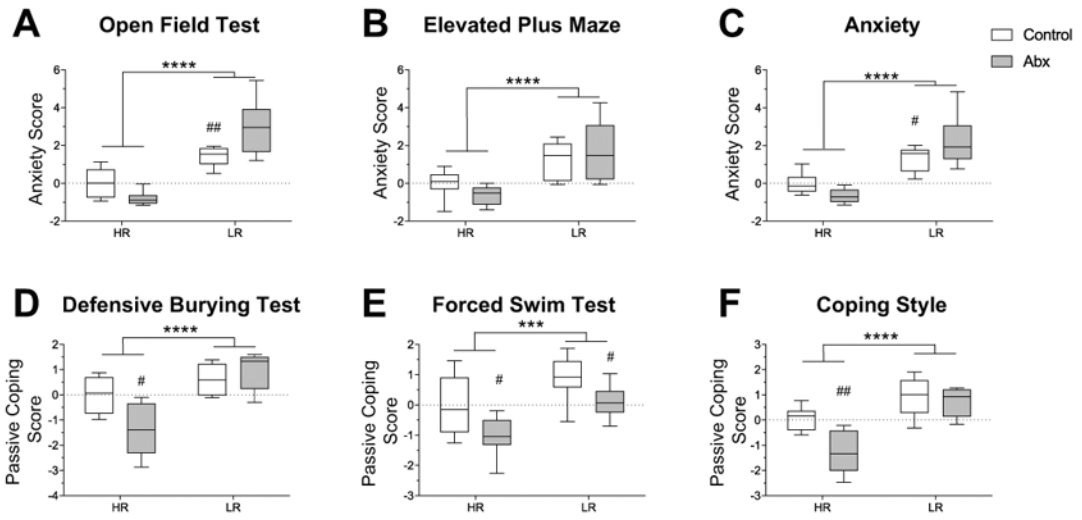

Figure 2: Antibiotic treatment alters HR/LR behavioral phenotypes.

LR rats overall exhibited greater anxiety-like behavior in the open field test (OFT) compared to HRs, and antibiotic treatment further increased anxiety-like behavior in LRs without affecting HRs (**A**). In the elevated plus maze (EPM), LRs exhibited greater anxiety-like behavior compared to HRs, with no significant effect of antibiotic treatment on either strain (**B**). Combinatorial analysis of behavioral measures across the OFT and EPM showed that LRs generally exhibited higher levels of anxiety-like behavior compared to HRs and that LRs' anxiety was further exacerbated with antibiotic treatment (**C**). In the defensive burying test, HRs displayed less passive coping (freezing behavior) compared to LRs. Antibiotic treatment further reduced passive coping in HRs, but did not impact LRs' behavior (**D**). In the Forced Swim Test (FST), LRs exhibited greater passive coping (immobility) compared to HRs. Antibiotic treatment had an antidepressant effect in the FST, leading to reduced passive coping in both HRs and LRs (**E**). When evaluating stress coping strategies across the defensive burying test and FST, LRs displayed more passive coping compared to HRs, and antibiotic treatment reduced passive coping in HRs, but not LRs (**F**). Asterisks (*) denote main effect of HR/LR phenotype (*** $p<0.001$, **** $p<0.0001$). Pound sign (#) denotes an effect of antibiotic treatment within HR/LR phenotype (# $p<0.05$, ## $p<0.01$).

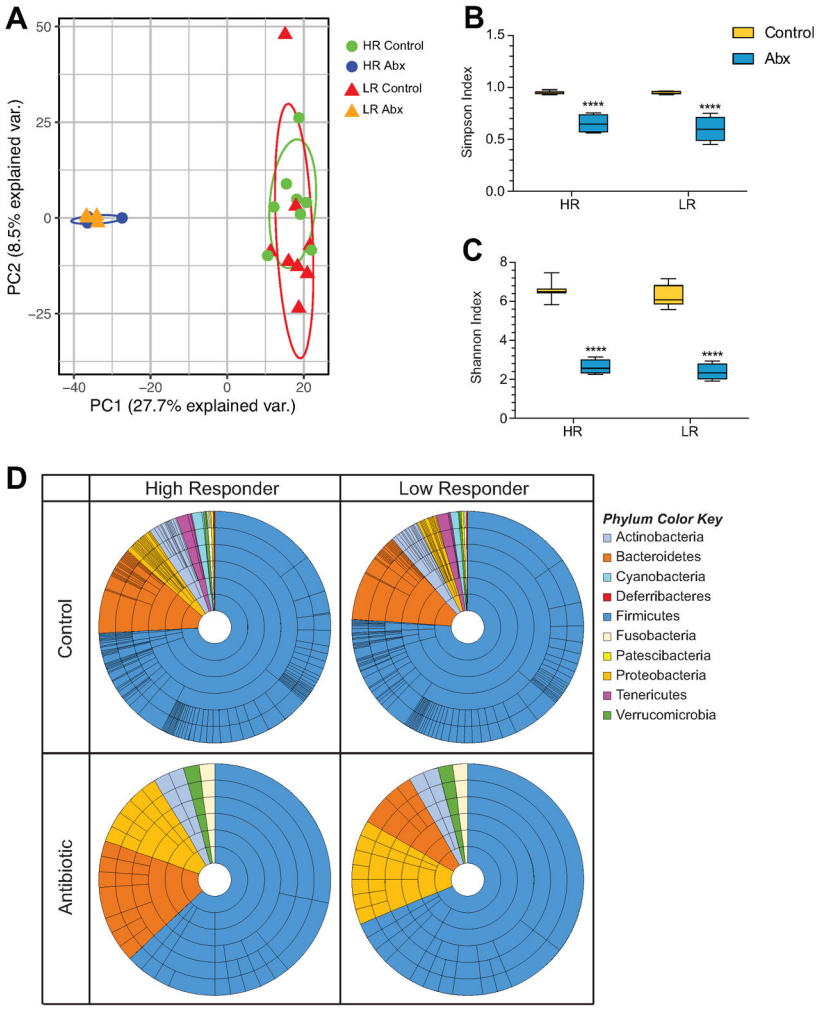

Figure 3: Antibiotic treatment alters HR/LR gut microbiota and reduces floral diversity.

Principle Component Analysis with Aitchison distance indicated robust antibiotic-induced variances in gut microbiota, but no HR/LR differences at baseline or after antibiotic treatment (**A**). Both Simpson and Shannon Indices were used to measure gut microbiota diversity, and while no overall HR/LR differences were observed, antibiotic treatment significantly reduced alpha diversity in both strains (**B-C**). Sunburst charts were generated using the OTUs present in each group to illustrate the microbe communities in HR controls, LR controls, antibiotic-treated HRs, and antibiotic-treated LRs (**D**). The phylogenetic tree is represented by phylum in the innermost rings through species on the outmost rings. ** denotes $p<0.01$, **** $p<0.0001$.

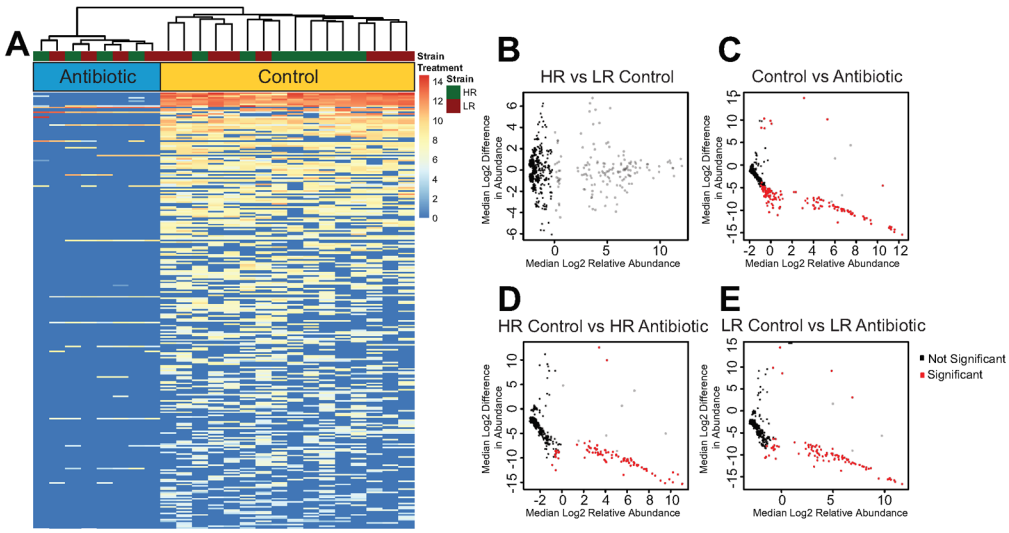

Figure 4: Effects of antibiotic treatment on gut microbiota.

Heatmap of altered microbiota OTUs in HR/LR rats (**A**). Volcano plots illustrating significant OTUs in control HRs versus LRs (**B**), control versus antibiotic-treated rats (**C**), HR controls versus antibiotic-treated HRs (**D**), and LR controls versus antibiotic-treated LRs (**E**).

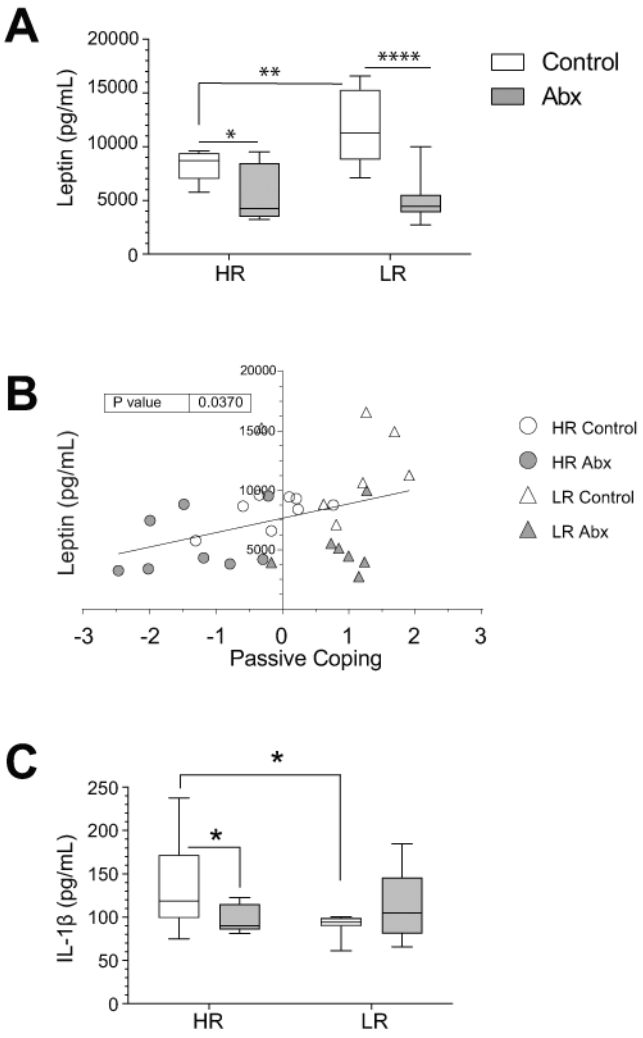

Figure 5: Antibiotic treatment reduced leptin serum levels.

Antibiotic treatment reduced serum leptin in both HR/LR (**A**), and leptin levels correlated with passive coping (**B**). Control HRs had higher levels of circulating IL-1β compared to control LRs (**C**). * denotes $p<0.05$, ** $p<0.01$, and **** $p<0.0001$.

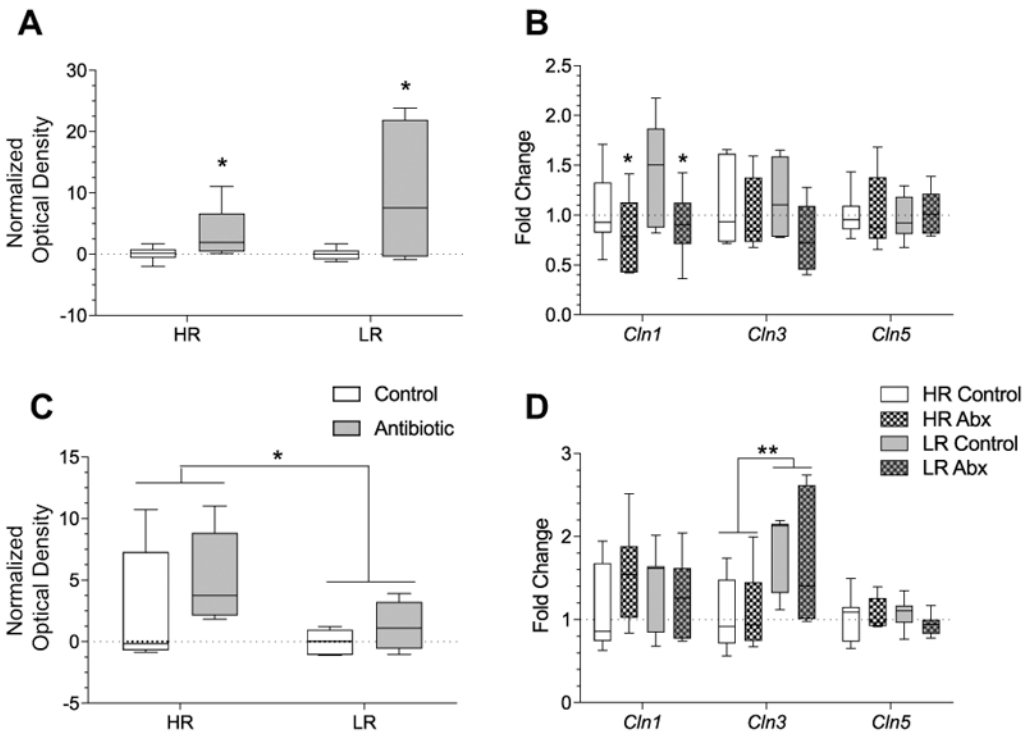

Figure 6: ***Antibiotic treatment increased BBB permeability in*** **basolateral amygdala** ***of HRs and LRs.***

Antibiotic treatment increased diffusion of Evans Blue into the brain from the vasculature into the basolateral amygdala of HRs and LRs (**A**). Antibiotic treatment reduced *Cldn1* mRNA levels in the basolateral amygdala of both HRs and LRs without affecting *Cldn3* or *Cldn5* (**B**). Compared to the LRs, HR animals demonstrated greater diffusion of Evans Blue from the vasculature into the ventral hippocampus. No effects of antibiotic treatment were detected (**C**). Both LR groups had higher mRNA expression of *Cldn3* compared to HRs. No strain or treatment differences were noted for *Cldn1* or *Cldn5* (**D**). * denotes $p<0.05$, ** $p<0.01$.

Table 1:

ANOVA tables for Figures 1B and 3A

Figure 1B*: Two-way Repeated Measures ANOVA analysis of animal weight*					
Variables	**df**	***sum of squares***	***MS***	***F (DFn, DFd)***	**p-value**
Group	3	11,694	3,898	F (3, 28) = 0.3388	0.7974
Time	16	107,323	6,708	F (16, 448) = 108.3	<0.0001
Group x Time Interaction	48	1,964	40.92	F (48, 448) = 0.6607	0.9612
Subject	28	322,116	11,504	F (28, 448) = 185.7	<0.0001
Residual	448	6.482	61.94		
Figure 3A*: PERMANOVA analysis of microbiome composition*					
Variables	**df**	***sum of squares***	***R^2***	***psuedo-F***	**p-value**
Group	1	0.1238	0.01909	1.075	0.274
Treatment	1	3.9639	0.61153	34.431	0.001
Interaction	1	0.0918	0.01415	0.797	0.436
Residual	20	2.3025	0.35522		
Total	23	6.482	1		

Results of the two-way repeated measures ANOVA for animal weight and PERMANOVA for microbiome composition.

Table 2:

Effects of antibiotic treatment on HR/LR behavior

Test	Behavioral Measure	Mean ± SEM				Significance (Two-way ANOVA)
		HR		LR		
		Control	*Abx*	*Control*	*Abx*	
Open Field	***Distance moved (cm)***	2859 ± 118.7	3276 ± 83.32	2067 ± 156.0	1365 ± 225.4 **	p=0.0012 Phenotype: F(1, 28) = 75.99, p<0.0001
	Percent time in center	5.397 ± 1.189	7.385 ± 1.049	2.152 ± 0.5149	0.4480 ± 0.2858	p=0.0396 Phenotype: F(1, 28) = 35.93, p<0.0001
	Fecal boli	1.125 ± 0.4407	0.6250 ± 0.3750	1.375 ± 0.2631	1.000 ± 0.3780	
Elevated Plus Maze	***Distance moved (cm)***	1518 ± 92.38	1854 ± 103.6	1180 ± 115.0	1205 ± 85.51	Phenotype: F(1, 28) = 24.49, p<0.0001
	Percent time in open arms	3.487 ± 2.448	5.264 ± 1.565	0.7244 ± 0.3313	2.822 ± 0.9836	
	Fecal boli	0.2500 ± 0.2500	0.0 ± 0.0	1.625 ± 0.5650	2.875 ± 0.9899	Phenotype: F(1, 28) = 13.27, p=0.0011
Forced Swim	***Percent time immobile***	47.04 ± 6.410	29.25 ± 3.201 *	63.79 ± 5.574	48.21 ± 2.231 *	p=0.0007 Treatment: F(1, 28) = 13.27, p=0.0013
	Percent time swimming	30.46 ± 4.475	43.88 ± 4.225 *	19.50 ± 2.889	28.21 ± 3.833	p=0.0020 Treatment: F(1, 28) = 8.036, p=0.0084
Defensive Burying	***Percent time immobile***	49.73 ± 7.643	27.12 ± 2.817 *	64.10 ± 5.795	73.31 ± 8.120	p=0.0216 Phenotype: F(1, 28) = 21.88, p<0.0001
	Percent time burying probe	3.594 ± 1.582	11.11 ± 3.767	6.339 ± 4.602	5.408 ± 3.825	
	Percent time in front (near probe)	4.748 ± 1.188	7.951 ± 1.394	2.930 ± 0.9995	0.9014 ± 0.2399	p=0.0209 Phenotype: F(1, 28) = 17.55, p=0.0003

Raw values of the behavioral data used to generate behavioral z-scores. The left column lists the behavioral tests included in this study, and the next column over lists the behavioral measures used to generate the z-scores for each behavior test. Data is presented as group mean ± SEM. * denotes effect of antibiotic treatment

* p<0.05

** p<0.01.