EARLY LIFE EXERCISE AND PREBIOTIC DIET MODULATE STRESS REACTIVE NEUROCIRCUITRY, INCREASE STRESS RESISTANCE, AND PROMOTE ADAPTIVE GUT MICROORGANISMS

By

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The final copy of this thesis has been examined by the signatories, and we find that both the content and the form meet acceptable presentation standards of scholarly work in the above mentioned discipline.

Abstract

Mika, Agnieszka (PhD., Integrative Physiology & Center for Neuroscience)

EARLY LIFE EXERCISE AND PREBIOTIC DIET MODULATE STRESS REACTIVE NEUROCIRCUITRY, INCREASE STRESS RESISTANCE, AND PROMOTE ADAPTIVE GUT MICROORGANISMS

Thesis directed by Professor Monika Fleshner, PhD

Early life is often described as period of vulnerability, as aversive events during this time lead to stress-related psychiatric disorders later in life. We demonstrate that early life is also a period of opportunity, as health promoting manipulations during this time can produce positive mental health outcomes that persist as the organism ages.

Exercise is one such positive manipulation. Using a rodent model, we have previously shown that six weeks of habitual exercise on running wheels protects against stress-induced anxiety and depressive-like behavior, and produces numerous neurobiological adaptations. These neural adaptations include plastic changes within the brain's serotonin circuits, which serve to attenuate stress-induced serotonergic dysregulation responsible for these behaviors. However, when exercise is begun in adulthood, these behavioral and neurobiological effects are short lived, and adults must continue to exercise in order to maintain mental health benefits. Here, we demonstrate that exercise begun earlier, during the developmentally sensitive juvenile period, can produce lasting protection against the behavioral consequences of stress. These effects persist even after wheel access is removed and the organism is no longer physically active, and are paralleled by persistent alterations in gene expression within serotonin circuits. In addition, durations of exercise shorter than those required by adult rats can produce lasting stress resistance.

Early life exercise produces robust adaptations in other physiological systems. We also demonstrate that exercise initiated during the juvenile period uniquely modulates gut microbial ecology by increasing bacteria and metabolites previously shown to benefit mental health. Furthermore, sedentary juvenile rats fed diets containing prebiotic fibers—fibers known to increase probiotic gut bacteria— are protected against stress-induced anxiety and depressive-like behavior later in life. Like exercise, early life prebiotic diet produces adaptations within stress-sensitive brain circuits. Moreover, oral antibiotics fed to exercising juvenile rats dampen the ability of exercise to protect against the behavioral consequences of stress. Our work highlights early life as a window of opportunity for lasting stress resistance, and suggests that gut microbes play a role in mediating these effects.

DEDICATION

The author gratefully acknowledges the following individuals for their assistance in the completion of this work.

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To my parents, especially my mom: Thank you for telling me that I can achieve anything I set my mind to.

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CHAPTER I

THE PERSISTENCE OF EXERCISE-INDUCED STRESS RESISTANCE DEPENDS ON THE DEVELOPMENTAL STAGE DURING WHICH EXERCISE IS INITIATED

In preparation for publication in Behavioral Neuroscience

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i. Abstract

Exercise reduces and protects against stress-associated psychiatric disorders in humans and psychiatric-like behaviors in rodents. We have demonstrated that rodent exercise on running wheels prevents the development of stress-induced learned helplessness (LH) behaviors, such as exaggerated fear and deficits in shuttle box escape learning. When exercise is initiated in young adulthood, these protective effects require 6 weeks of exercise to develop and dissipate between 15 and 25 days following removal of wheel access. However, if exercise is initiated earlier in life, it may be capable of producing stress protection that lasts longer and develops more quickly. Thus, we first examined the persistence of exercise-induced stress resistance in adult (PND 70) and juvenile (PND 24) male, Fisher (F344) rats. Rats were housed with a running wheel or remained sedentary for 6 weeks, after which all wheels were rendered immobile. Then, either immediately following exercise cessation, 15 or 25 days later, rats underwent inescapable stress (IS) or no stress and subsequent testing for LH behaviors. Exercise begun in the juvenile period protected rats against LH at all time points. Conversely, exercise in adulthood only protected rats immediately following exercise, as rats were once again vulnerable to IS-induced LH behaviors 15 and 25 days following wheel access removal. To explore the neural mechanisms underlying the lasting adaptations produced by juvenile exercise, we examined gene expression for 5-HT1A presynaptic inhibitory autoreceptors (5-HT1AR) within the dorsal raphe nucleus (DRN). We have previously shown that exercise increases gene expression for 5-HT1AR in the DRN, an adaptation that may help constrain IS-induced serotonergic dysregulation and prevent LH. Brains were collected from adult and juvenile runner and sedentary rats both immediately following 6 weeks of exercise or and 25 days later. *In situ* hybridization revealed that early life exercise increases 5-HT1AR mRNA in select subregions of the DRN both immediately and 25 days

following exercise cessation, and may protect against age-associated 5-HT1AR mRNA decline. Finally, we explored whether shorter periods of exercise in early life were sufficient to produce long lasting stress resistance. Juvenile (PND 24) male rats either ran or remained sedentary for three weeks, after which all wheels were rendered immobile. 25 days following cessation of exercise, all rats underwent IS or no stress and subsequent testing for LH behaviors. Three week of juvenile-onset exercise was sufficient to produce long lasting stress protection. These results suggest that when exercise is initiated during early sensitive developmental periods, it can alter the trajectory of brain development to protect against stress in adulthood.

ii. Introduction

The potent health benefits of exercise in humans include the ability to alleviate depression and anxiety (Carek et al., 2011; Mason & Powell, 1985; Mortazavi et al., 2012; Paluska & Schwenk, 2000). Using a rodent model, we have shown that adult rats given access to running wheels for six weeks are protected against anxiety and depressive-like behaviors produced by acute inescapable stress (IS; Greenwood & Fleshner, 2008; Greenwood et al., 2005a; Greenwood et al., 2003; Greenwood et al., 2012). IS exposure produces learned helplessness (LH; reviewed in Maier & Watkins, 2005), a collection of behaviors that resemble certain features of human depression and anxiety (Greenwood & Fleshner, 2008); for instance, exaggerated shock-elicited fear following IS in rodents can be a measure of anxiety-like behavior, while increased escape learning latencies can be measures of instrumental learning deficits, commonly demonstrated in patients with depression.

Exercise can protect against exaggerated fear and escape learning deficits following IS by producing adaptations in brain circuits that underlie LH behaviors. IS produces LH by hyper-activating dorsal raphe nucleus (DRN) serotonin (5-HT) neurons (Grahn et al., 1999; Maswood et al., 1998). These neurons are then temporarily sensitized, so that mildly aversive stimuli can elicit exaggerated amounts of 5-HT within the DRN as well as DRN projection regions. IS produces this sensitized state by desensitizing inhibitory 5-HT1A autoreceptors (5-HT1AR). When stimulated, these presynaptic somatodendritic autoreceptors exert negative feedback within the DRN by reducing 5-HT production and cell firing (Chen & Penington, 1996; Valdizan et al., 2010). The intense IS-induced activation of DRN neurons transiently desensitizes 5-HT1ARs (Rozeske et al., 2011), removing this brake, and allowing for excessive 5-HT release in projection regions mediating LH behaviors, like the amygdala, important for exaggerated shock

elicited freezing, and the dorsal striatum, involved in instrumental escape learning. Exaggerated 5-HT within these regions during behavioral testing elicits these LH behaviors (Amat et al., 1998a, 1998b). Exercise, however, can prevent this cascade of events. Indeed, we've also shown that six weeks of exercise can dampen stress-induced cfos induction within 5-HT DRN neurons (Greenwood et al., 2003; Greenwood et al., 2005a) and prevent IS-induced 5-HT elevations in DRN projection sites (Clark et al., 2015). Though the exact mechanism by which exercise protects against IS is still elusive, we've demonstrated increased 5-HT1AR mRNA in the dorsal aspect of the rostro-mid DRN after six weeks of exercise in adult rats (Greenwood et al., 2005b; Greenwood et al., 2003; Loughridge et al., 2013). According to Greenwood and Fleshner (2011), one way increased 5-HT1AR mRNA can prevent LH is through increases in functional receptor proteins that help to restrict DRN hyper-activation during IS. Taken together, regular exercise can produce adaptations in the 5-HT system that enable a stress-resistant phenotype.

When exercise is initiated in late adolescence or adulthood, rodents must exercise for durations greater than three weeks, as six but not three weeks can protect against IS-induced LH behaviors and DRN hyper-activation (Greenwood et al., 2005a). Furthermore, these protective effects are transient; once wheel access is removed, the adult rat is once again susceptible to IS-induced LH within 15-25 days of exercise cessation (Greenwood et al., 2012). It is, however, possible that these limitations depend on the developmental period during which exercise occurs. Other groups demonstrate that the beneficial effects of exercise may be more robust and persistent if initiated during early developmental stages. Hopkins et al., (2011) reported that early life exercise can upregulate brain-derived neurotrophic factor (BDNF) protein and enhance certain types of memory later in life, while adults who were physically active during childhood,

compared to those who were sedentary, are significantly less likely to suffer from depression (McKercher et al., 2014).

Despite these data, the effects of exercise during sensitive developmental periods are understudied. In contrast, the maladaptive behavioral and neurobiological effects of aversive early life events, such as stress, have been explored extensively. Results from such human (Culpin et al., 2015; Heim & Nemeroff, 2001; Heim et al., 2004; Rincon-Cortes & Sullivan, 2014; Whitesell et al., 2009) and rodent (Bledsoe et al., 2011; Brydges et al., 2012; Lyttle et al., 2015; Tsoory et al., 2007) studies demonstrate that stressor exposure is more likely to produce permanent changes in mood, behavior and brain function if experienced earlier in life. However, just as negative events impinge on proper brain development, we hypothesize that positive early life manipulations, like exercise, can take advantage of the plastic, labile state of the developing brain and produce lasting beneficial changes.

In the present study, we sought to determine whether the stress-protective behavioral and neuro-plastic effects of exercise persist longer when exercise is initiated during the juvenile period, beginning at PND 24, versus adulthood (PND 70). Furthermore, we also examined whether shorter periods of exercise (3 weeks) were sufficient to produce lasting protection against the behavioral consequences of stress.

iii. Materials and methods

a. Rats and wheel running

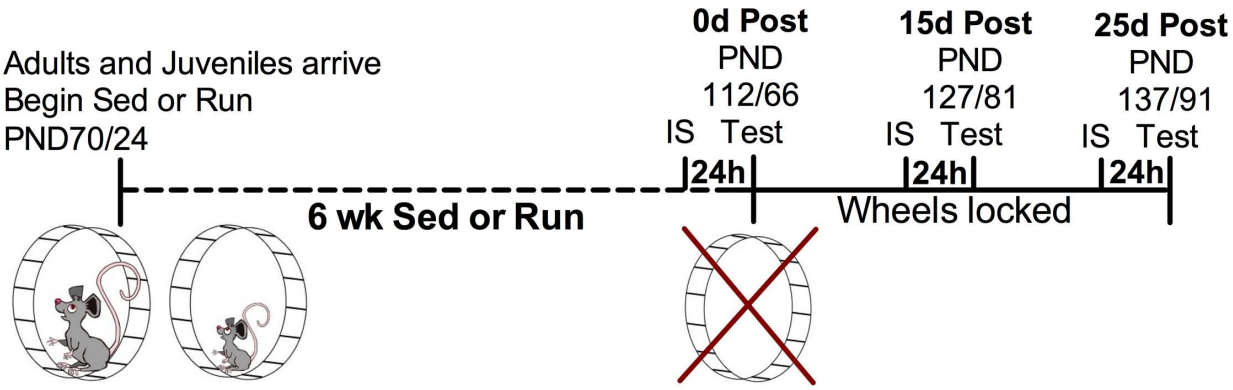
Adult (PND 70) and juvenile (PND 24) male Fischer 344 rats (Envigo; Indianapolis, IN) were pair-housed on a 12:12 h light/ dark cycle in humidity and temperature (22°C) controlled housing. Pair housing was employed due to the stressful nature of single housing juveniles (Takemoto et al., 1975). Sedentary rats were housed in standard Nalgene Plexiglas cages (45cm

× 25.2cm × 14.7cm), and runner rats were housed in a standard cage equipped with a running wheel (Mini Mitter Instruments). Wheel revolutions were digitally recorded daily, with Vital View software (Mini Mitter, Bend, OR, USA). Running distance was calculated by multiplying the number of wheel revolutions by the circumference of the running wheel (1.081 m). All rats had ad libitum access to food and water for the duration of each study, and all rats were weighed weekly. The University of Colorado Animal Care and Use Committee approved all protocols for these experiments, and care was taken to ensure minimal discomfort during all procedures.

b. Experimental design

Experiment 1: To examine how exercise in early life affects the persistence of exercise-induced behavioral stress resistance. Adult (PND 70) and juvenile (PND 24) rats began their respective exercise or sedentary conditions immediately upon arrival. Following 6 weeks of exercise, all running wheels were rendered immobile with metal stakes. Following cessation of exercise, separate cohorts of rats were exposed to inescapable stress (IS) followed by behavioral testing at three separate time points: one day prior to exercise cessation, 14 days following exercise cessation, and 24 days following exercise cessation. Given that behavioral testing for shock-elicited freezing and shuttle box escape learning occurred 24 hours following IS, behavioral testing for learned helplessness (LH) behaviors occurred either immediately, 15 days or 25 days following exercise cessation, respectively. Thus, exercised rats were forced to remain sedentary for either 0, 15 or 25 days prior to behavioral testing. At each time point, juvenile and adult sedentary and runner rats were randomly assigned into counterbalanced IS or home cage control (HCC) groups, yielding a 2 (age) X 2 (exercise) X 2 (stress) X 3 (time point) design.

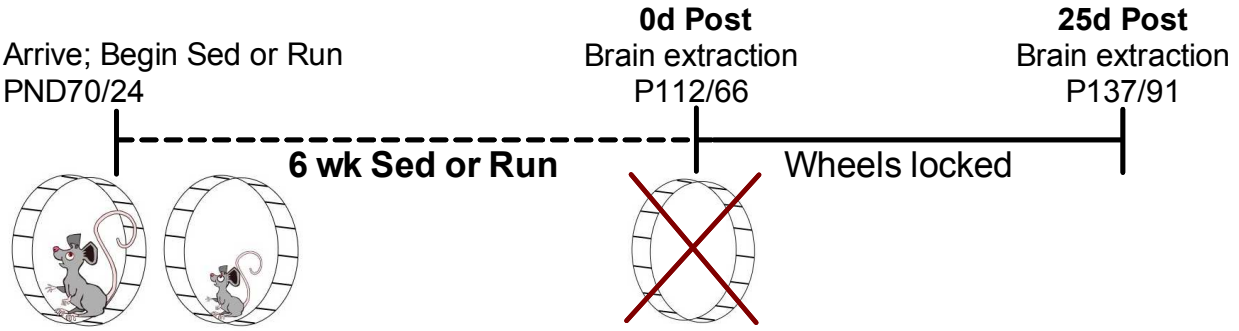
Figure 1



Experimental timeline. Upon arrival, juvenile and adult male Fischer 344 rats were immediately allowed access to running wheels or remained in standard sedentary cages. Following 6 weeks of exercise, wheels were rendered immobile with metal stakes. Separate cohorts of rats were exposed to inescapable stress (IS) followed by behavioral testing for LH behaviors, at three separate time points, so that behavioral testing occurred either immediately, 15 days or 25 days following exercise cessation. At each time point, adult and juvenile sedentary and runner rats were either exposed to IS or remained in their home cage, then subsequently underwent testing for shock-elicited freezing and shuttle box escape learning 24 hours later.

Experiment 2: To examine whether exercise in early life produces persistence alteration in gene expression within 5-HT circuits underlying IS-induced LH behaviors. Six weeks of exercise in adult rats can increase 5-HT1AR mRNA in the dorsal aspect of the rostral and mid dorsal raphe nucleus (Greenwood et al., 2003), but the persistence of these adaptations, and whether exercise earlier in life can produce more robust or lasting adaptations is still unknown. In a similar design, adult (PND 70) and juvenile (PND 24) male, Fisher (F344) rats were allowed access to a running wheel or remained sedentary for 6 weeks. Either immediately or 25 days following cessation of exercise, separate cohorts of rats were sacrificed and brains were collected to examine gene expression using *in situ* hybridization.

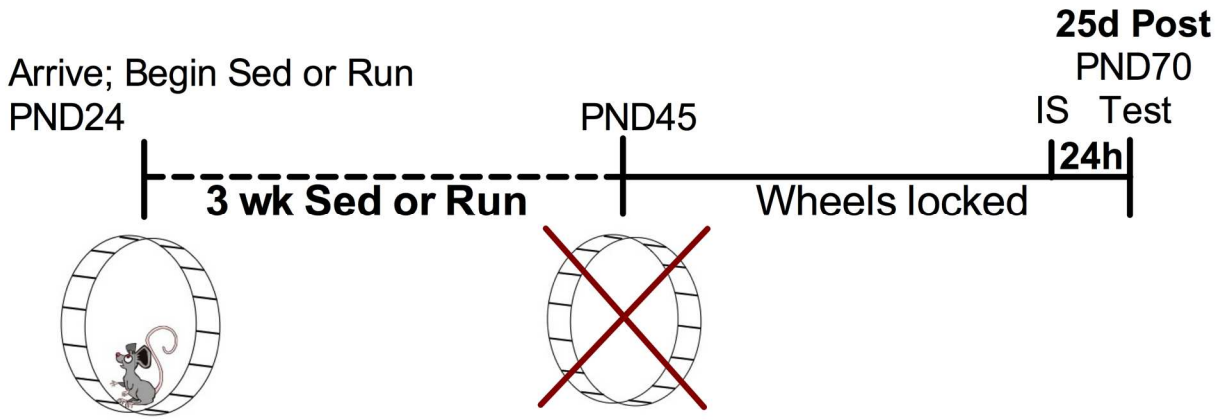
Figure 2



Experimental timeline. Upon arrival, juvenile and adult male Fischer 344 rats were immediately allowed access to running wheels or remained in standard sedentary cages. Following 6 weeks of exercise, wheels were rendered immobile with metal stakes. Either immediately or 25 days following exercise cessation, separate cohorts of rats were sacrificed, and brains and plasma were extracted for later analysis.

Experiment 3: To examine whether shorter periods of exercise in early life are capable for producing persistent behavioral stress resistance. We have previously demonstrated that rodents must exercise for durations greater than three weeks, as six but not three weeks can protect against IS-induced LH behaviors and DRN hyperactivation (Greenwood et al., 2005a). However, whether a similar timeline is necessary for juvenile rats is still unknown. Juvenile, male, Fischer F344 rats were allowed access to a running wheel or remained sedentary for 3 weeks. After 3 weeks, the running wheels were locked and all rats remained sedentary for 25 days. 24 days following exercise cessation, rats were exposed to inescapable stress (IS) followed by behavioral testing for shock-elicited freezing and shuttle box escape deficits occurred 24 hours later, so that rats underwent testing for LH behaviors 25 days following cessation of exercise.

Figure 3



Experimental timeline. Upon arrival, juvenile male Fischer 344 rats were immediately allowed access to running wheels or remained in standard sedentary cages. Following 3 weeks of exercise, wheels were rendered immobile with metal stakes. 25 days following exercise cessation, rats were exposed to inescapable stress (IS) followed by behavioral testing for LH behaviors. At this time, juvenile sedentary and runner rats were either exposed to IS or remained in their home cage, then subsequently underwent testing for shock-elicited freezing and shuttle box escape learning 24 hours later.

c. Inescapable stress

During the inactive (light) cycle from 0800 to 1000, rats were restrained in Broome-style Plexiglas tubes (23.4 cm in length and 7.0 cm in diameter) with their tails exposed for electrode attachment. Over a 2-hour period, rats receive 100, 1 mA inescapable tail shocks administered via local electrodes, at variable intervals (VI of 60 s). Shock intensity increased from 1.0 to 1.5mA after the first 50 shocks to account for stress-induced analgesia. Rats were returned to their home cage immediately following IS termination.

d. Behavioral testing

24 hours after IS, rats were assessed for anxiety and depressive-like behaviors with the shock-elicited freezing and shuttle box escape behavioral tasks.

Shock-elicited freezing and subsequently, shuttle box escape deficits were assessed in the same testing session, with each session lasting approximately 1 hour during the inactive cycle from 0800 to 1200. Following previously established protocols (Greenwood et al., 2003), rats were placed in shuttle boxes (50.8cm × 25.4cm × 30.48cm, Coulbourn Instruments, Whitehall, PA) and allowed 10 minutes of exploration. During this time, rats were hand-scored every 10 seconds as freezing (i.e. no movement other than respiration) or not freezing by a blind experimenter. Following the 10-minute exploratory period, rats received 2 fixed ratio 1 (FR-1) foot shocks (0.1 mA, 60 s ITI). Each shock continued until the rat crossed from one side of the shuttle box to the other; latencies to cross were recorded. Subsequently following the last FR-1, rats were again assessed for freezing for 20 minutes, to examine conditioned fear to environmental cues associated with the shuttle box. Following the shock-elicited freezing task, the shuttle box escape task was begun. During this time, rats received 25 fixed ratio 2 (FR-2) foot shocks (0.6 mA, 60 s ITI). Each shock was continuously administered (for a maximum of 30

seconds), until the rat crossed from one side of the shuttle box to the other twice; latencies to cross were recorded. If a rat failed to cross twice within 30 seconds, a latency score of 30 was given, and the shock was terminated.

e. Sacrifice and Tissue Collection

Rats in experiment 2 were sacrificed using rapid decapitation. Brains were extracted and frozen for later analysis of gene expression, and the thymus, spleen and adrenal gland were removed and weighed.

f. Preputial separation assessment

In the third experiment, a subset of rats was assessed for preputial separation, or separation of the prepuce from the glans penis, an anatomical marker for puberty onset (Korenbrot et al., 1977). This was does one time, when rats reached PND 45 at the end of the three week running period, to assess what developmental period rats were in at this time. This involved applying firm, gentle pressure at the base of the genitalia and visually assessing whether the prepuce had begun to physically detach from the glans penis.

g. In situ hybridization

Following sacrifice in experiment 2, brains were removed and immediately flash frozen in isopentane cooled with dry ice (-20 C; 4 minutes), and sliced at 10 µm on a cryostat (CM 1850, Leica Microsystems, Nussloch, Germany). Brains were stored at -80 °C prior to being sectioned; sectioning occurred at -24 °C. Rostral-caudal sections of the DRN were be collected and thaw-mounted onto FisherBrand Colorfrost ® Plus slides (Fisher Scientific Company LLC, Denver, Co, USA). Tissues sections were then again stored at -80 °C prior to *in situ* hybridization.

Following previous protocols (Mika et al., 2015a), slides containing brain tissue were fixed in 4% paraformaldehyde for 1 hour, then washed 3 times in 2X saline-sodium citrate (SSC) buffer, acetylated with 0.25% acetic anhydride containing 0.1 M triethanolamine for 10 minutes, and dehydrated with graded ethanol. Simultaneously during tissue processing, riboprobes for 5-HT1AR (911 mer, 333-1243 coding region) were transcribed with radioactive Uridine 5'-triphosphate UTP ([35S-UTP]; Perkin-Elmer, Waltham, MA, USA). After verification of radioactive labeling, the riboprobes were added to 50% hybridization buffer, comprised of 50% high-grade formamide, 10% dextran sulfate, 3X SSC, 1X Denhardt's solution, 0.2 mg/mL yeast tRNA, and 0.05 M sodium phosphate (pH 7.4). 70 µl of the buffer mixture was then applied each slide containing sections of the DRN. Slides were subsequently cover slipped, and incubated overnight at 55 °C in humid chambers. The following day, slides were washed 3 times in 2X SSC and incubated for 1 hour in RNase A (200 ug/mL), rinsed in graded concentrations of SSC, washed in 0.1X SSC at 65 C for 1 hour, and dehydrated in graded ethanol. After drying, slides were placed in light-tight autoradiography cassettes and exposed to X-ray films (Kodak or Biomax-MR) for 2 weeks.

h. Image Analysis for in situ Hybridization

Briefly, levels of 5-HT1AR mRNA were analyzed by computer-assisted optical densitometry in accordance to previously established protocols. Images of each brain section were captured digitally (CCD camera, model XC-77; Sony, Tokyo, Japan), and the relative optical density of the x-ray film was determined using Scion Image Version 4.0 software (Scion, Frederick, MD, USA). Signal above background in each brain slice was determined automatically using a macro written for this purpose. For each section, an area was sampled over white matter or over a non-tissue containing portion of the slide, and the signal threshold was

determined as mean grey value of background +3.5 standard deviations; only pixels with grey values above these criteria were included in the analysis. Results were expressed as a mean integrated density, which is reflective of both the signal intensity and the number of pixels above assigned background (mean signal above background multiplied by number of pixels above background). Templates for each region were made to ensure that equivalent areas are analyzed between animals. Quantification of 5-HT1AR mRNA in the DRN occurred at the following levels (Paxinos and Watson, 1998): rostral, -7.40mm to -7.64mm; mid, -7.80mm to -8.00mm; caudal, -8.30 to -8.50mm. 2-4 tissue sections were analyzed per subject per each approximate rostrocaudal level. These values were then averaged together to give a mean integrated density at each level for each subject.

i. Statistical analyses

Body weight was analyzed using 2 (age) x 2 (exercise) x time repeated measures ANOVAs; separate analyses were conducted for body weight measured during exercise versus in the weeks following exercise cessation. Running distances were summed nightly during the active cycle, and further summed to produce total distance per week. These values were divided by two to estimate distance per rat, and analyzed using a 2 (age) x 6 (week 1- 6) or 3 (week 1-3) repeated measures ANOVA. Tissues were analyzed using a 2 (age) x 2 (time point) x 2 (exercise) ANOVA. Mean shock-elicited freezing scores and mean shuttle box escape scores were analyzed using a 2 (age) x 2 (stress) x 4 (time since exercise cessation: sed, run at 0d, run at 15d, run at 25d) ANOVA. Shock-elicited freezing scores were also analyzed across time: scores were collapsed into 10, 2-minute blocks and analyzed using a 2 (stress) x 4 (time since exercise cessation: sed, run at 0d, run at 15d, run at 25d) x 10 (time) repeated measures ANOVA. Similarly, shuttle box escape latencies were averaged into 5 blocks of 5 trials each, and analyzed

using a 2 (age) x 2 (exercise) x 5 (time) repeated measures ANOVA. Given the anatomical topography of the rostral and mid DRN, these regions were analyzed together; in the dorsal, ventral and lateral DRN, the rostral and mid subregions were analyzed using a 2 (subregion; rostral, mid) x 2 (age) x 2 (time point) x 2 (exercise) ANOVA; in the dorsal and ventral DRN, the caudal subregion was analyzed using a 2 (age) x 2 (time point) x 2 (exercise) ANOVA.

iv. Results

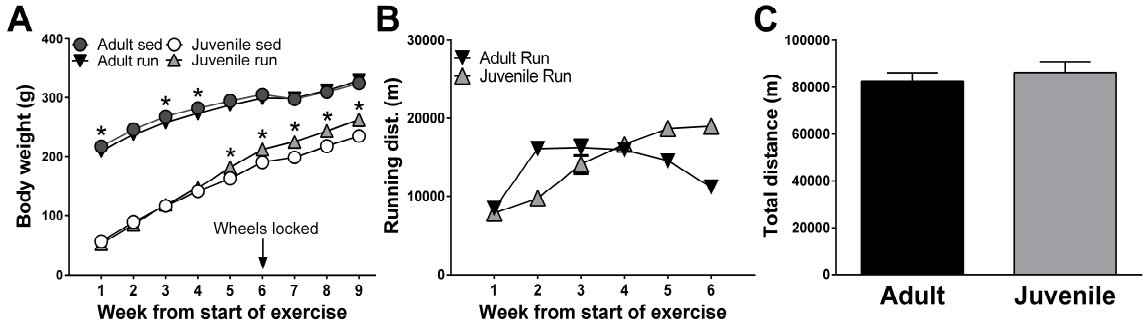
a. Body weight & running distance

Body weight. All rats, regardless of age, gained weight over the course of the experiment, both during exercise ($F(5,760)=2648.528$; $p<0.0001$) and after exercise cessation ($F(5,70)=398.515$; $p<0.0001$; Figure 4A). In addition, juvenile rats weighed significantly less than adult rats throughout exercise ($F(1,760)=2387.327$; $p<0.0001$), as well as following exercise cessation (Age $F(1,70)=465.431$; $p<0.0001$). A significant main effect of exercise was not detected during exercise, though a time x age x exercise interaction ($F(5,760)=9.258$; $p<0.0001$) demonstrated that adult runners gained less weight than adult sedentary rats at certain time points during exercise (see graphs for post hocs), while in contrast, juvenile runners weighed more than juvenile sedentary rats toward the end of exercise. This pattern in juvenile rats persisted after exercise cessation, as revealed by a main effect of exercise ($F(1,70)=13.347$; $p=0.0005$), and an age x exercise ($F(1,70)=9.961$; $p=0.00240$) and time x age $F(5,70)=15.453$; $p=0.0002$) interaction.

Running distance. Running patterns for both adult and juvenile runners changed over time ($F(5,430)=105.834$; $p<0.0001$). Although adult versus juvenile rats exhibited different running patterns throughout the six week period with adults running more in the beginning and

decreasing toward the end, while juveniles ran less in the beginning and more in the end, no significant main effect of age was detected ($F(1,430)=0.597$; $p=0.4420$).

Figure 4



Body weight and running distance. A) Body weight across the experiment. B) Running distance, measured as average total distance per week, estimated per rat, across six weeks of exercise. C) Total distance run summed across six weeks for adult and juvenile runners. Data are represented as mean ± SEM. N/grp: body weight: juvie sed:16, adult sed: 14, juvie run: 40, adult run: 50; running distance: Juvie run: 38 (one cage excluded for malfunctioning wheel), adult run: 50.

b. Early life exercise produced longer lasting protection against stress-induced learned helplessness behaviors

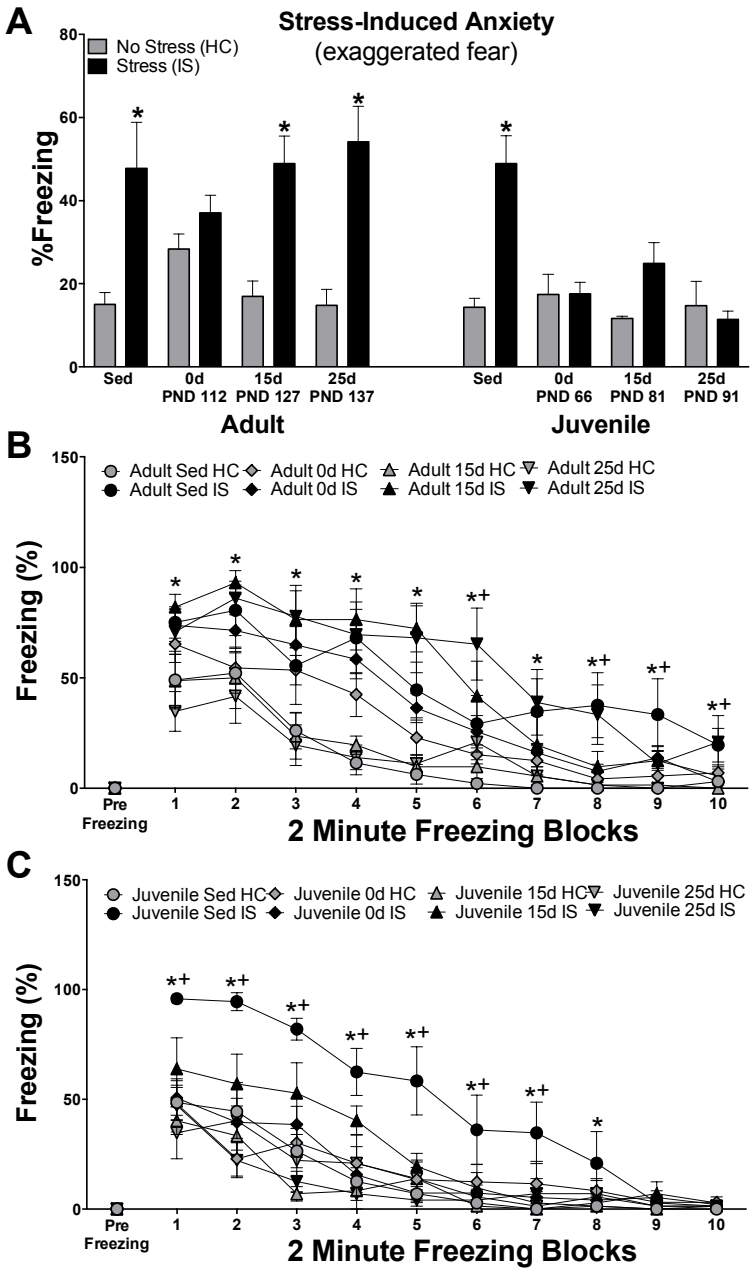
Shock-elicited Freezing. Figure 5A depicts average freezing for the 20-minute testing session. ANOVA revealed that juveniles froze less than adults overall ($F(1,100)=23.807$; $p<0.0001$), and IS significantly increased freezing overall ($F(1,100)=56.466$; $p<0.0001$). A main effect of time since exercise cessation, along with an age x time since exercise cessation x stress interaction ($F(3,100)=3.080$; $p=0.0309$) revealed that the effects of IS depend upon both age and time since exercise cessation. In sedentary as well as previously physically active adults tested either 15 or 25 days following cessation of exercise, IS increased freezing levels. Adult runners tested immediately (0d) following exercise cessation were the only ones protected from IS-induced increases in shock-elicited freezing. In contrast, IS significantly increased shock-elicited freezing in sedentary juvenile rats only; no significant effects due to IS were detected in juvenile runners immediately, 15 days nor 25 days following exercise cessation.

Figure 5B depict freezing levels for adult groups across the 20-minute testing session. Repeated measures ANOVA demonstrates that freezing levels decreased across the testing session ($F(9,504)=75.321$; $p<0.0001$), and IS increased freezing levels ($F(1,504)=47.702$; $p<0.0001$). An effect of time since exercise cessation was not detected ($F(3,504)=0.088$; $p=0.9665$), however, a time x time since exercise cessation ($F(27,504)=1.975$; $p=0.0027$) and a time x stress ($F(9,504)=5.941$; $p<0.0001$) interaction revealed that adult runners tested immediately (0d) following exercise cessation were the only ones protected from IS-induced increases in shock-elicited freezing.

Figure 5C depicts freezing levels for juvenile groups across the 20-minute testing session. Repeated measures ANOVA demonstrates that freezing levels decreased across time

($F(9,396)=69$; $p<0.0001$), and that IS increased freezing levels ($F(1,396)=13.602$; $p=0.0006$). A main effect of time since exercise cessation ($F(3,396)=6.636$; $p=0.0009$) and a time x time since exercise cessation x stress interaction ($F(27,396)=2.290$; $p=0.0003$) revealed that IS significantly increased freezing levels in sedentary juvenile rats only; rats with a history of exercise, regardless of time since cessation of exercise, did not exhibit increased freezing levels following IS.

Figure 5



Early life exercise produced longer lasting protection against stress-induced exaggerated freezing relative to adult exercise. A) Average percent time spent freezing, during the 20 min testing session. Stress and non-stressed, time-matched sedentary controls are averaged together and labelled as "Sed." All other time points represent stress and non-stressed runners, either immediately (0d), 15d, or 25d following exercise cessation. B) 2-minute blocks of shock-elicited freezing across the 20 min testing session, for adults and C) juveniles at each testing time point. Data are represented as mean ± SEM. */+ $p < 0.05$. * indicates a difference between HC and IS, + indicates difference between sedentary IS runner IS. N: 6-14/grp.

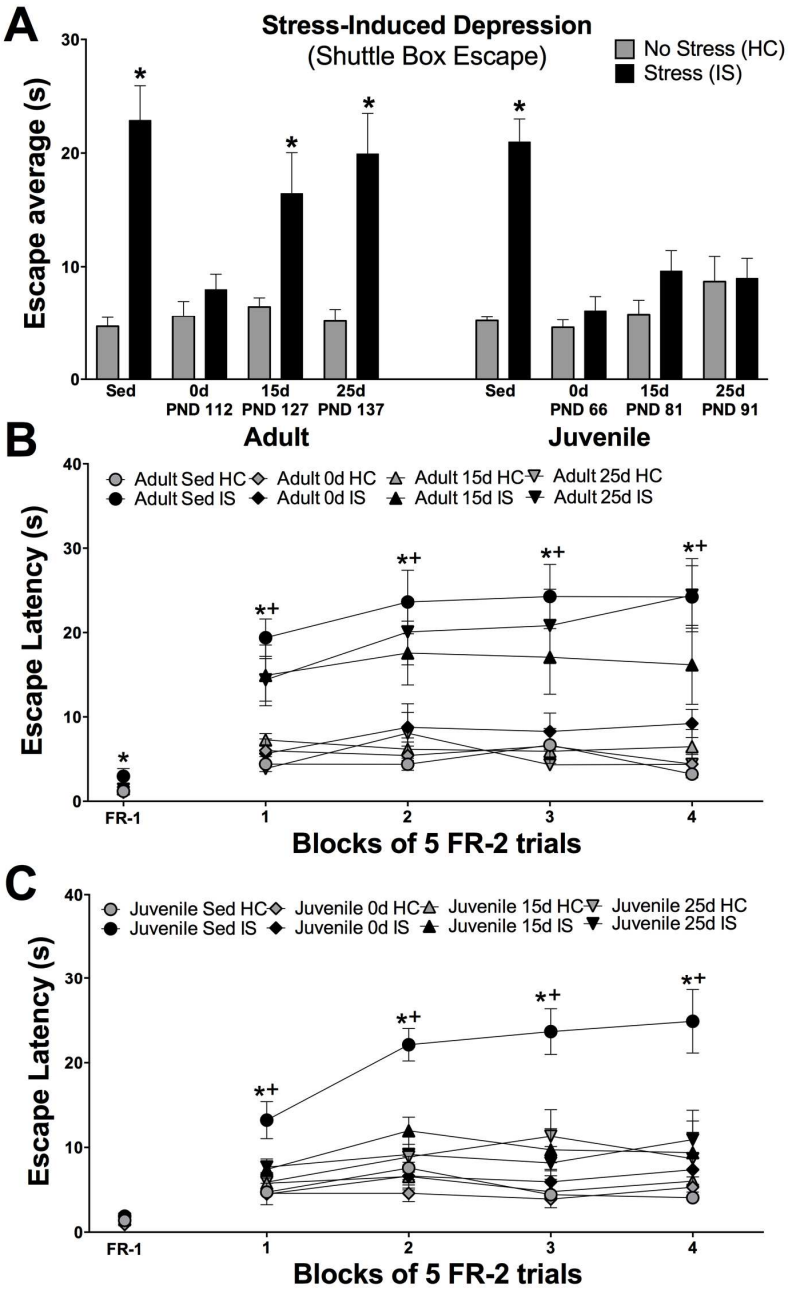
Shuttle box escape. Figure 6A depicts average escape latency. Similarly, ANOVA revealed that juvenile rats exhibited faster escape latencies overall, compared with adults ($F(1,98)=6.631$; $p=0.0115$), and that IS increased latency to escape ($F(1,98)=80.648$; $p<0.0001$). A main effect of time since exercise cessation ($F(3,98)=12.511$; $p<0.0001$) and age x stress ($F(1,98)=10.341$; $p=0.0018$) as well as day x stress ($F(3,98)=12.455$; $p<0.0001$) interactions and subsequent post hoc comparisons further revealed that exercise protected adult rats from shuttle box escape deficits only at the immediate time point (0d). In contrast, all juvenile rats with an exercise history were protected from IS-induced increases in shuttle box escape latencies.

Figure 6B depicts escape latencies for adult groups across the testing session. Differences in escape latencies between groups were detected in the FR-1 trial; both time since exercise cessation ($F(3,54)=5.281$; $p=0.0029$), and stress ($F(1,54)=7.348$; $p=0.009$) impacted FR-1 escape latencies, and a time since exercise cessation x stress ($F(3,54)=5.626$; $p=0.002$) plus subsequent post hocs revealed that the sedentary adult rats exposed to IS exhibited significantly longer FR-1 escape latencies than their sedentary, non-stressed counter-parts. Repeated measures ANOVA revealed that FR-2 escape latencies increased across time ($F(3,165)=4.538$; $p=0.0044$), and that IS increased escape latencies ($F(1,165)=60.676$; $p<0.0001$). A main effect of time since exercise cessation ($F(3,165)=6.052$; $p=0.0012$) and a time x stress interaction ($F(3,165)=5.134$; $p=0.0020$) further revealed that adult runners were only protected if tested immediately following exercise cessation, as IS increased escape latency for adults with a history of exercise when they were tested 15 or 25 days since exercise cessation (see graph for specific post hoc comparisons).

Figure 6C depicts escape latencies for juvenile runners across the testing session. No differences in escape latencies between groups were detected in the FR-1 trial. Repeated measures ANOVA revealed that FR-2 escape latencies increased across time ($F(3,129)=7.964$;

$p<0.0001$), and IS increased escape latencies overall ($F(1,129)=25.777$; $p<0.0001$). A main effect of time since exercise cessation ($F(3,129)=9.709$; $p<0.0001$), as well as a time x day x stress interaction ($F(9,129)=2.372$; $p=0.0163$) further reveled that IS increased latencies to escape in sedentary juvenile rats only; juveniles with a history of exercise were protected from IS-induced increases in escape latency at all time points, regardless of time since exercise cessation.

Figure 6



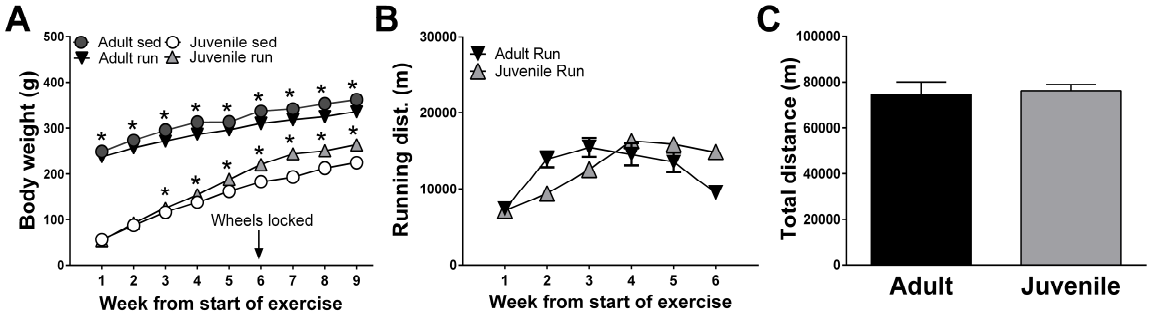
Early life exercise produced longer lasting protection against stress-induced shuttle box escape deficits relative to adult exercise. Stress and non-stressed, time-matched sedentary controls are averaged together and labelled as "Sed." All other time points represent stress and non-stressed runners, either immediately (0d), 15d, or 25d following exercise cessation. A) Average escape latency across the entire testing session, for adults and juveniles at each time point. B) Escape latencies across time, in 5 blocks of 5 trials. Data are represented as mean ± SEM. */+ $p < 0.05$. * indicates a difference between HC and IS, + indicates a difference between sedentary IS and runner IS. N: 6-14/grp.

c. Body weight and running distance

Body weight. All rats, regardless of age, gained weight over the course of the experiment, both during exercise ($F(5,490)=1622.769$; $p<0.0001$; Figure 7A), as well as following cessation of exercise ($F(2,100)=38.033$; $p=0.0001$), though juvenile rats weighed significantly less than adult rats throughout the duration of exercise ($F(1,490)=4431.547$; $p<0.0001$), as well as in the weeks following exercise ($F(1,100)=371.503$; $p<0.0001$). Again, a significant main effect of exercise was absent during exercise, however, a time x age x exercise interaction was detected ($F(5,490)=20.639$; $p<0.0001$), in that adult runners weighed less than their sedentary counterparts, while juvenile runners weighed more than their sedentary counterparts (see graphs for post hoc comparisons at each specific time point). This pattern persisted following cessation of exercise, with juvenile runners continuing to weigh more and adult runners continuing to weigh less than their sedentary counterparts, as demonstrated by an age x exercise interaction ($F(2,100)=51.872$; $p<0.0001$) and subsequent post hocs.

Running distance. Similar to the first experiment, running patterns for both adult and juvenile runners changed over time ($F(5,220)=54.828$; $p<0.0001$). Adult versus juvenile rats exhibited different running patterns throughout the six-week period, with adults running more in the beginning of the exercise period and decreasing toward the end while juveniles exhibited the opposite pattern. However, no significant main effect of age was detected ($F(1,220)=0.074$; $p=0.7867$).

Figure 7



Body weight and running distance. A) Body weight across the experiment. B) Running distance, measured as average total distance per week, estimated per rat, across six weeks of exercise. C) total distance summed across six weeks of exercise. Data are represented as mean ± SEM. N/grp: body weight: 24-26/grp; running distance: 22-24/grp.

d. Exercise did not affect physiological indicators of chronic stress activation

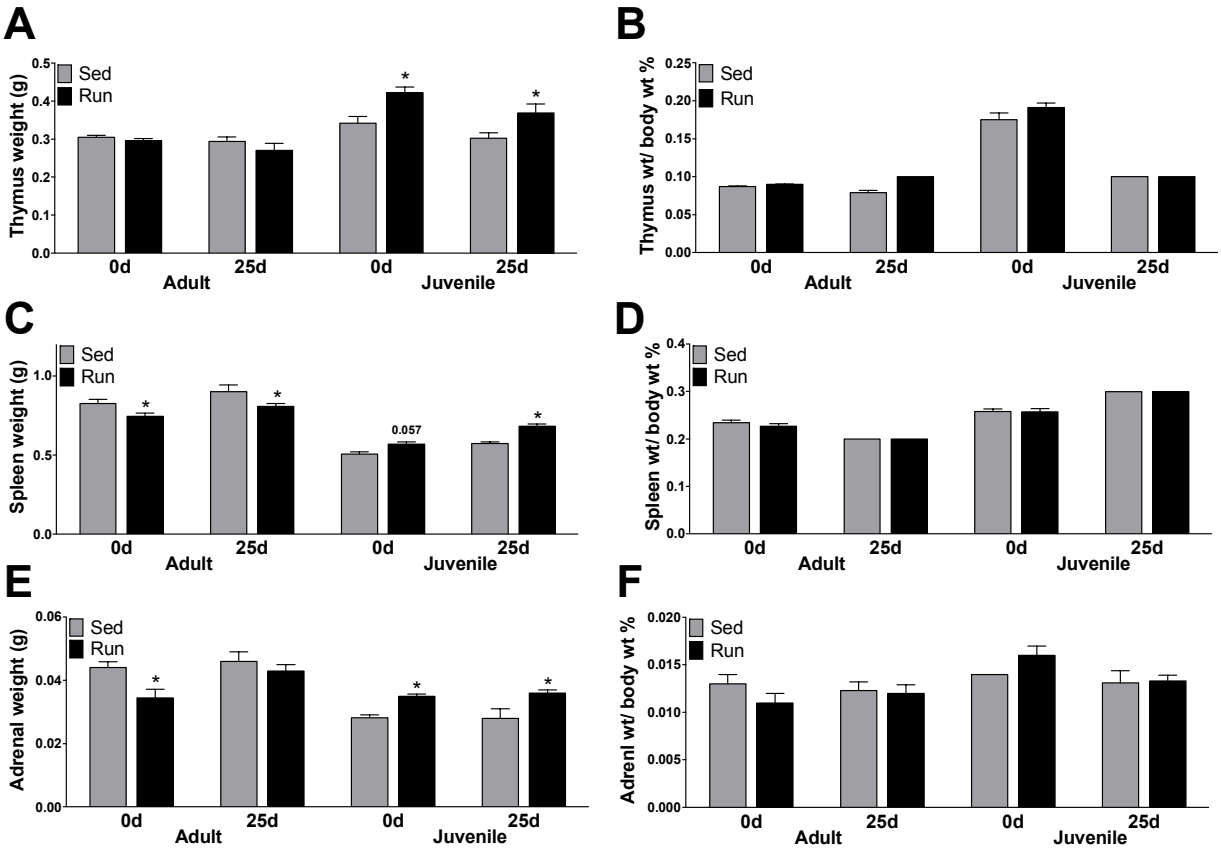
Figure 8 represents various tissues taken from a subset of rats from each group at each time point. Thymic weight differed by age ($F(1,40)=39.060$; $p<0.0001$), exercise ($F(1,40)=6.769$; $p=0.0129$) as well as time since exercise cessation ($F(1,40)=8.555$; $p=0.0057$). An age x exercise ($F(1,40)=16.749$; $p=0.0002$) interaction was detected, and it appeared that early life exercise increased thymic weight immediately and 25 days following exercise cessation (Figure 8A). However, thymic weight corrected by body weight demonstrated a different pattern of results (Figure 8B). Here, thymic weight still differed by age ($F(1,40)=352.395$; $p<0.0001$) and time point ($F(1,40)=45.855$; $p<0.0001$) but not by exercise ($F(1,40)=0.404$; $p=0.5288$). An age x time since exercise cessation interaction ($F(1,40)=15.351$; $p=0.0003$) revealed that early life exercise slightly increased thymic weight at the immediate time point only ($p=0.06$).

Spleen weight differed by age ($F(1,40)=226.591$; $p<0.0001$) as well as time since exercise cessation ($F(1,40)=25.416$; $p<0.0001$). An age x exercise ($F(1,40)=30.092$; $p<0.0001$) interaction was detected, and it appeared that adult exercise decreased spleen weight immediately and 25 days following exercise cessation, while early life exercise increased it (Figure 8C). However, spleen weight corrected by body weight demonstrated a different pattern of results (Figure 8D). Here, spleen weight still differed by age ($F(1,40)=31.198$; $p<0.0001$).

Adrenal weight differed by age ($F(1,40)=46.163$; $p<0.0001$) as well as time since exercise cessation ($F(1,40)=4.027$; $p=0.0516$). An age x exercise ($F(1,40)=20.268$; $p<0.0001$) interaction was detected, and it appeared that adult exercise decreased adrenal weight immediately, while early life exercise increased adrenal weight both immediately and 25 days following exercise cessation (Figure 8C). However, adrenal weight corrected by body weight demonstrated a different pattern of results (Figure 8D). Here, adrenal weight still different by age

($F(1,40)=17.155$; $p=0.0002$) and an age x time point ($F(1,40)=5.298$; $p=0.0266$) interaction demonstrated a trend toward a decrease following exercise in adulthood, immediately following cessation of exercise ($p= 0.08$).

Figure 8



Thymus, spleen and adrenal weight. Impact of juvenile and adult onset exercise on thymus, spleen and adrenal weight, both raw (A, C, E) and represented as a percentage of total body weight (B, D, F), respectively, either immediately or 25d following cessation of exercise. Data are represented as mean ± SEM. * $p < 0.05$. N: 6/grp.

e. 5-HT1AR mRNA expression in the DRN.

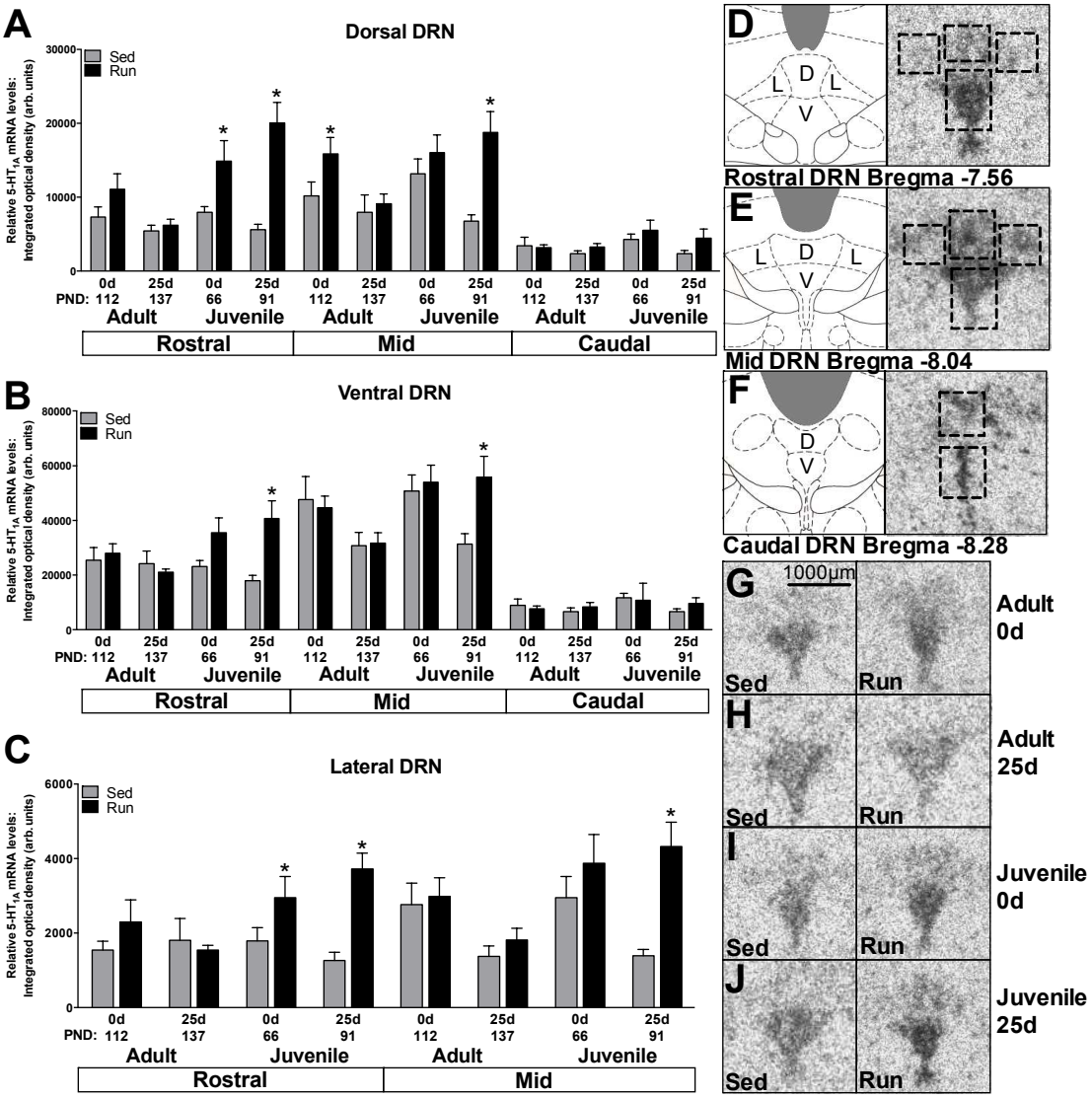
Figure 9A depicts 5-HT1AR mRNA expression in the dorsal DRN, across rostral, mid and caudal subregions. Expression differed between rostral and mid subregions ($F(1,160)=6.228$; $p=0.0136$). Furthermore, in the rostral and mid DRN, juvenile rats exhibited greater 5-HT1AR mRNA expression overall ($F(1,160)=15.274$; $p=0.0001$) and 5-HT1AR mRNA expression was increased following exercise overall ($F(1,160)=38.308$; $p<0.0001$). Within these two regions, 5-HT1AR mRNA expression also differed by time since exercise cessation ($F(1,160)=4.621$; $p=0.0331$). An age x exercise ($F(1,160)=10.477$; $p=0.0015$) and an age x time since exercise cessation x exercise ($F(1,160)=9.939$; $p=0.0019$) interaction and subsequent post hocs revealed that in the rostral DRN, exercise increased 5-HT1AR mRNA expression in juvenile rats only, both immediately and 25 days following exercise cessation. In the mid DRN, exercise increased 5-HT1AR mRNA expression in the adult rats at the immediate time point only, while in juvenile rats, 5-HT1AR mRNA expression was increased specifically 25 days following cessation of exercise. No significant effects were detected in the caudal DRN.

Figure 9B depicts 5-HT1AR mRNA expression in the ventral DRN, across rostral, mid and caudal subregions. Similarly, expression was higher in the mid subregion relative to rostral ($F(1,160)=40.345$; $p<0.0001$). Furthermore, in the rostral and mid DRN, juvenile rats exhibited greater 5-HT1AR mRNA expression overall ($F(1,160)=7.405$; $p=0.0072$), exercise increased overall expression ($F(1,160)=8.603$; $p=0.0038$), and expression differed by time since exercise cessation ($F(1,160)=7.358$; $p=0.0074$). An age x exercise interaction ($F(1,160)=10.155$; $p=0.0017$) and subsequent post hocs revealed that in the rostral DRN, exercise increased 5-HT1AR mRNA expression in juvenile rats only, specifically 25 days following cessation of exercise. Similarly in the mid DRN, 5-HT1AR mRNA expression was increased in juvenile rats

only, specifically 25 days following cessation of exercise. No significant effects were detected in the caudal DRN.

Figure 9C depicts 5-HT1AR mRNA expression in the lateral DRN, across rostral and mid subregions. Similarly, expression was higher in the mid subregion relative to rostral ($F(1,150)=5.576$; $p=0.0195$). Furthermore, in the rostral and mid DRN, juvenile rats exhibited greater 5-HT1AR mRNA expression overall ($F(1,150)=10.149$; $p=0.0018$). Furthermore, exercise increased overall expression ($F(1,150)=20.002$; $p<0.0001$), and expression differed by time since exercise cessation ($F(1,150)=4.131$; $p=0.0439$). An age x exercise interaction ($F(1,150)=10.703$; $p=0.0013$), age x time since exercise cessation x exercise interaction ($F(1,150)=4.492$; $p=0.0357$) and subsequent post hocs revealed that in the rostral lateral DRN, exercise increased 5-HT1AR mRNA expression in juvenile rats only, at both time points while in the mid lateral DRN, juvenile exercise increased 5-HT1AR mRNA expression 25 days following exercise cessation only.

Figure 9



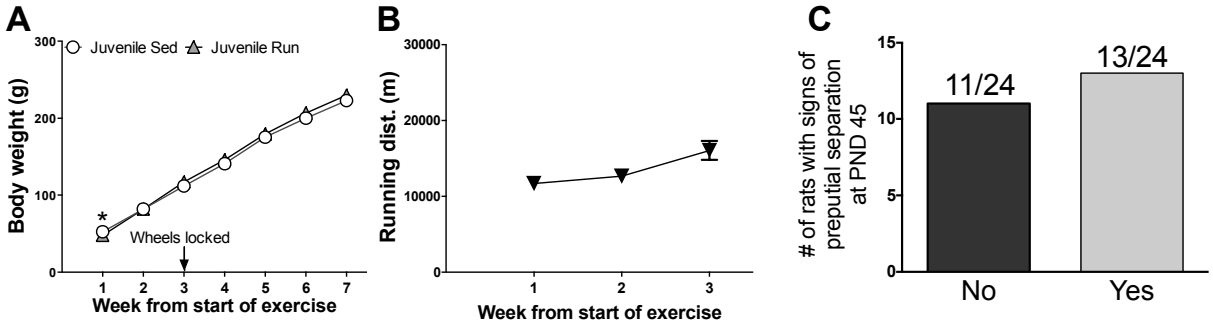
Early life exercise produced long lasting increases in 5-HT1AR mRNA expression within the DRN. A) 5-HT1AR mRNA expression in the dorsal aspect of the DRN, across rostral, mid, and caudal regions. B) 5-HT1AR mRNA expression in the ventral aspect of the DRN, across rostral, mid, and caudal regions. C) 5-HT1AR mRNA expression in the rostral and mid lateral wings of the DRN. D-F) Atlas images and corresponding autoradiographs showing in situ hybridization of 5-HT1AR in the rostral, mid, and caudal DRN. Areas outlined on the autoradiographs indicate regions sampled for quantification. G-J) Representative images of the mid DRN for each group. Data are represented as mean ± SEM: * $p < 0.05$. * N/grp: rostral: 9-12/grp; mid: 11-12/grp; caudal: 9-12/grp; rostral lateral: 9-12/grp; mid lateral: 11-12/grp.

f. Body weight and running distance

Body weight. Juvenile rats gained weight over the course of the experiment, regardless of exercise condition ($F(6,264)=2894.153$; $p<0.0001$; Figure 10A). A time x exercise interaction ($F(5,264)=2.976$; $p=0.0079$) revealed that in the first week of exercise, juvenile sedentary rats weighted more than juvenile runners.

Running distance. Figure 10B represents average total distance per week, estimated per rat, across three weeks of exercise. Running patterns for juvenile rats increased steadily throughout the three weeks ($F(2,46)=21.545$; $p<0.0001$).

Figure 10



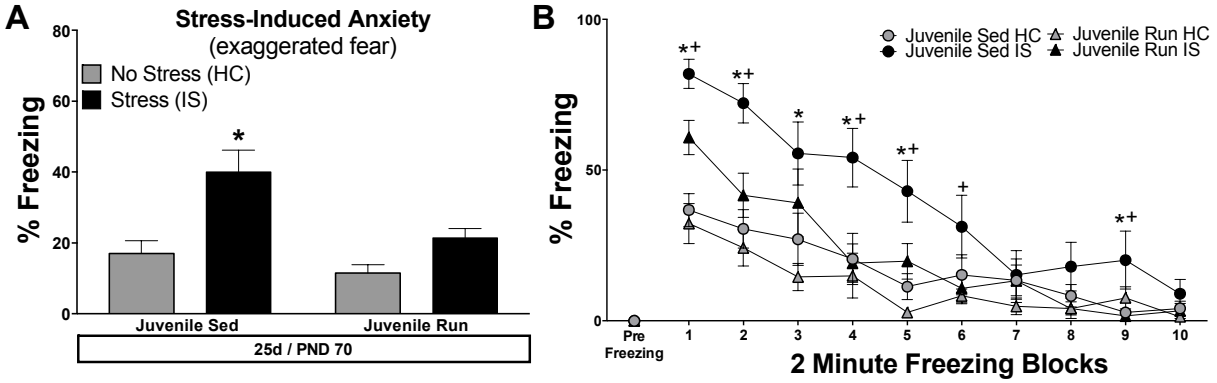
Body weight, running distance, and preputial separation. A) Body weight across the experiment. B) Running distance, measured as average total distance per week, estimated per rat, across three weeks of exercise. C) Number of rats, out of 24, demonstrating preputial separation, a marker for pubertal maturation. Data for A & B are represented as mean ± SEM; while data for C are represented at total number of rats demonstrating preputial separation, from a subset of rats examined. N/grp: body weight: 22-24/grp; running distance: 24/grp.

g. Three weeks of exercise is sufficient to produce long lasting protection against stress-induced learned helplessness

Figure 11A depicts average freezing levels for juvenile runner and sedentary rats tested 25 days following cessation of exercise. Overall, IS increased freezing levels ($F(1,42)=16.040$; $p=0.0002$), while exercise decreased freezing levels ($F(1,42)=8.661$; $p=0.0053$). Post hoc comparisons revealed that IS increased freezing levels in sedentary rats only.

Figure 11B depicts freezing levels across the 20-minute testing session. Freezing levels decreased overall across the testing session ($F(9,378)=38.355$; $p<0.0001$) and a time x stress interaction ($F(9,378)=5.511$; $p<0.0001$) revealed that IS increased freezing levels in the juvenile sedentary rats only, especially during the beginning portion of the testing session.

Figure 11

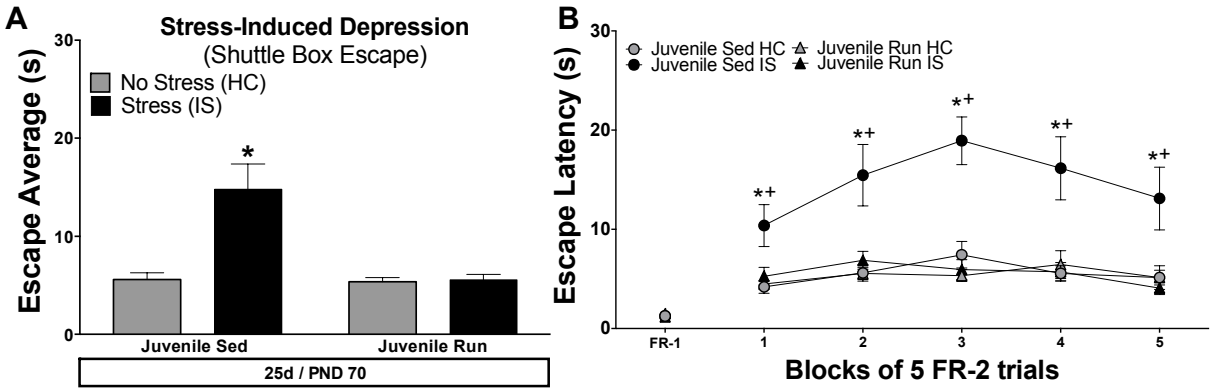


Three weeks of early life exercise produced long term protection against stress-induced shock-elicited freezing. A) Average percent time spent freezing. B) Shock-elicited freezing across the 20 min testing session, in 2-minute blocks. Data are represented as mean ± SEM. */+$p < 0.05$. * indicates a difference between HC and IS, + indicates a difference between difference between sedentary IS and runner IS. N: 10-12/grp.

Shuttle box escape. Figure 12A depicts average escape latency in juvenile runner and sedentary rats tested 25 days following cessation of exercise. Overall, IS increased escape latencies ($F(1,43)=11.155$; $p=0.0017$); and exercise decreased escape latencies ($F(1,43)=11.241$; $p=0.0017$). An exercise x stress interaction ($F(1,43)=10.286$; $p=0.0025$) and subsequent post hocs revealed that IS increased average escape latencies in sedentary rats only.

Figure 12B depicts escape latencies for each group across the testing session. No differences in escape latencies between groups were detected in the FR-1 trial. Overall, IS increased FR-2 escape latencies while exercise decreased them, and an exercise x stress interaction and subsequent post hocs revealed that IS increased average escape latencies in sedentary rats only. Escape latencies differed across time ($F(4,172)=7.990$; $p<0.0001$).

Figure 12



Three weeks of early life exercise produced long term protection against stress-induced shuttle box escape deficits. A) average escape latency across the entire testing session. B) escape latencies across time, in 5 blocks of 5 trials. Data are represented as mean ± SEM. */+$p < 0.05$. * indicates a difference between HC and IS, + indicates a difference between sedentary IS and runner IS. N: 10-12/grp.

v. Discussion

The current data demonstrate that exercise in early life is capable of producing long-lasting behavioral stress resistance and corresponding adaptations in gene expression, relative to the short lived effects produced by exercise initiated in adulthood. Specifically, exercise initiated during the juvenile period protected rats against IS-induced anxiety and depressive-like behavior and produced accompanying increases in 5-HT1AR mRNA within the DRN—both adaptations were present 25 days following removal of wheel access. Furthermore, the behavioral effects showed no signs of attenuation, indicating that perhaps stress resistance produced by early life exercise can last beyond 25 days. We also demonstrated that shorter durations of exercise, compared with those previously shown to be required by young adults/ adolescents (Greenwood et al., 2005a), produced persistent stress resistance. Here, as little as 3 weeks of juvenile-onset exercise produced lasting protection against IS-induced LH behaviors. Conversely, 6 weeks of adult-onset exercise was insufficient to produce lasting protection against LH and persistent 5-HT1AR mRNA adaptations. When exercise was initiated at PND 70, the behavioral effects dissipated sometime within 15 days following removal of wheel access while adaptations in gene expression dissipated within 25 days. These data collectively demonstrate that when exercise is initiated in early life, brief periods of exercise can produce enduring adaptations that protect the organism against the negative mental health effects of stress. Conversely, when exercise is initiated outside of sensitive developmental periods, developmentally mature adult rats need to continue to exercise in order to sustain stress resistance, and require longer periods of exercise in order to produce these effects in the first place (Greenwood et al., 2005a).

We did not demonstrate an increase in exaggerated shock-elicited freezing behavior in non-stressed physically active rats following removal of wheel access, as was previously demonstrated by Greenwood et al. (2012). In the aforementioned study, these adverse effects

were likely due to forced exercise withdrawal. In the present study, the social enrichment from pair-housing conditions may have mitigated these negative behavioral effects.

Both juvenile and adult onset exercise increased 5-HT1AR mRNA expression in certain subregions of the DRN; however, significant increases due to exercise in adulthood were restricted to the dorsal mid subregion, and only occurred immediately following exercise cessation. In contrast, juvenile onset exercise produced both immediate and long lasting increases in 5-HT1AR mRNA expression in multiple DRN subregions. 5-HT1ARs within the DRN exert important negative feedback against excessive 5-HT release. LH behaviors following IS are caused by IS-induced hyperactivation and sensitization of 5-HT DRN neurons, due to the desensitization of 5-HT1AR. Prior work from our lab demonstrates that six weeks of exercise in adolescent/ young adult rats can increase 5-HT1AR mRNA in the DRN (Greenwood et al., 2005b; Greenwood et al., 2003), and attenuate IS-induced hyper-activation of 5-HT DRN neurons (Greenwood et al., 2003). Thus, exercise-induced increases in 5-HT1AR mRNA may buffer against the IS-induced DRN hyper-activation.

Interestingly, increases in 5-HT1AR mRNA immediately following early life exercise were only apparent in the rostral dorsal and rostral lateral DRN; in the dorsal mid and rostral ventral, mid ventral, and mid lateral DRN, increases due to exercise were seen 25 days following cessation of exercise only. Here, the data patterns indicate that group differences are mainly due to decreases in basal mRNA expression in sedentary controls across time points, rather than exercise-induced increases in 5-HT1AR mRNA. Thus, it appears that early life exercise may be able to protect against age-associated decline in 5-HT1AR mRNA expression.

Surprisingly, juvenile onset exercise altered 5-HT1AR mRNA expression within the ventral DRN while adult onset exercise did not. A lack of an effect within the ventral DRN is in

line with previous reports (Greenwood et al., 2005b; Greenwood et al., 2003), which demonstrate no significant differences due to exercise in the ventral aspect of the DRN in adolescent /young adult rats. The current study thus demonstrates that exercise produces different patterns of expression, of differing stability, based upon when exercise is initiated.

We also measured thymus, spleen and adrenal weights to ensure that exercise did not elicit a stress response in either age group. Thymus, adrenal and spleen weights are physiological indicators of chronic stress; if exercise were to chronically activate stress responsive systems, we would expect to see adrenal hypertrophy, thymic involution due to chronic hypothalamic-pituitary-adrenal axis stimulation, and decreased spleen weight due to either chronic or acute sympathetic nervous system activation. No differences in tissue weights were detected, following body weight correction.

Despite exhibiting distinct running patterns, overall running distance between adults and juveniles was not different overall. Thus, the unique behavioral and neurobiological effects produced by early life exercise are unlikely to be attributed to different running distances between age groups. In addition, previous work from our lab (Greenwood et al., 2005a; Greenwood et al., 2003; Greenwood et al., 2013) demonstrates that running distances are not related to behavior, suggesting that greater running distance does not influence the degree of exercise-induced stress resistance. Furthermore, the behavioral responses of non-stressed physically active rats did not differ from non-stressed sedentary rats, so it is also unlikely that long lasting changes in physical fitness in the juvenile runner rats are attributable to long lasting stress resistance.

In our first two experiments, juvenile rats that ran for six weeks likely ran throughout developmental periods that are roughly analogous to human childhood, pubertal development,

adolescence and early adulthood. However, in our third experiment, exercise was mostly restricted to three weeks in early development. Since three weeks of exercise in early life was sufficient to produce lasting behavioral stress resistance, we sought to determine the developmental stage of our juvenile runner and sedentary rats at PND 45, the age juvenile rats reached following three weeks of exercise. The age of transition from the juvenile period into adolescence is discrepant amongst rat strains (Andersen, 2003; Romijn et al., 1991; Sengupta, 2013); Andersen (2003) asserts that the rise in gonadal hormones characteristic of puberty occurs sometime around PND 30, whereas other work (Sengupta, 2013) contends that can it occur around PND 40. Our own observations of preputial separation in F344 male rats from the current data demonstrate that about 54% of juvenile rats sampled had begun showing signs of preputial separation at PND 45. Preputial separation is an external marker for puberty onset (Korenbrot et al., 1977), and thus a useful for marking the transition into adolescence. Thus, we approximate that in F344 male rats, sexual maturation of reproductive organs indicative of pubertal maturation likely occurs sometime between PND 40-50. Thus, for the majority of our rats in our third experiment, the three weeks of exercise was indeed restricted to the juvenile period. It's important to note that puberty is not synonymous with adolescence—puberty specifically refers to the period during which the organism reaches sexual maturation, whereas adolescence is a transitional stage between childhood and adulthood, during which the organism achieves emotional and behavioral maturation, in addition to sexual maturation (Sisk & Zehr, 2005). Pubertal maturation is closely associated with the brain and behavioral maturation events that are characteristic of adolescence, and thus preputial separation (Korenbrot et al., 1977) can be used to mark those transitory stages.

Converging lines of evidence from our data and prior studies suggests that the pre-pubertal juvenile period may be a sensitive period for exercise to produce lasting and robust stress resistance. Here, both three and six weeks of exercise initiated in the juvenile period are sufficient to produce lasting stress resistance. In contrast, prior work demonstrated when exercise was initiated approximately in adolescence, between PND 42-49, the protective effects of exercise dissipated sometime between 15 and 25 days following cessation of exercise, and furthermore, three weeks were insufficient to produce even immediate behavioral stress resistance (Greenwood et al., 2005a). In addition, results from early life stress studies demonstrate that stressor exposure is more likely to produce permanent changes in brain function, particularly in limbic circuits governing mood and behavior, if it occurs prior to puberty (Bledsoe et al., 2011; Brydges et al., 2012; Lyttle et al., 2015; Tsoory et al., 2007). In contrast, when stress is initiated close to or after puberty, longer lasting maladaptive consequence are less likely to occur (Tsoory & Richter-Levin, 2006).

Prior work also suggests that the juvenile period may be a developmentally sensitive time serotonergic systems. Broadly, mammalian brain development is viewed as being comprised by two major waves of plasticity during which axons and synapses are rapidly overproduced then pruned (Andersen, 2003; Crews et al., 2007). These processes occur immediately before birth then again throughout childhood and adolescence (Baird et al., 1999). Importantly, these maturational events do not occur simultaneously throughout the brain, and have very different developmental trajectories depending upon the brain region, circuit, and neurotransmitter system in question (Andersen, 2003; Casey et al., 2000; Giedd et al., 1999; Giedd et al., 1996; Sisk & Zehr, 2005; Zehr et al., 2006). Animal studies have demonstrated regional re-organization of 5-HT systems throughout the juvenile period. Although for the majority of 5-HT neuronal

development, distribution and innervation reaches completion within prenatal and early postnatal periods, region-specific alterations in serotonin receptor expression continue throughout early childhood/ juvenile development (reviewed in (Booij et al., 2015)). The specific developmental pattern of serotonin expression depends upon the particular protein and brain region in question. While studies examining the developmental trajectory of 5-HT1AR within the DRN are lacking, one rodent study found that 5-HT1AR expression levels increased by 50% within the dorsal aspect of the DRN between PND 14- PND 28 (Sidor et al., 2010). Other work investigating 5-HT1AR expression across development in other brain regions has shown that the majority of limbic structures experience fluctuations within receptor expression, coupling and binding throughout the juvenile development specifically (Booij et al., 2015). Although human studies investigating serotonin system development are also sparse, one human study demonstrated global alterations in 5-HT synthesis showed that 5-HT synthesis rates increase until about age 5, then start to decrease, and reach stable, adult levels by age 14 (Chugani et al., 1999). This work collectively demonstrates that the development of the 5-HT system continues throughout the juvenile period, and may be sensitive to environmental manipulation.

In the current study, we did not investigate the mechanisms by which exercise in early life could be producing lasting adaptations in gene expression. However, there are several potential avenues to explore for future research. For instance, exercise in early life could be producing plastic changes in brain regions regulating the DRN 5-HT circuit. Greenwood and Fleshner (2011) provide a potential mechanism by which exercise-induced activation of the noradrenergic LC can increase 5-HT1AR gene expression. Exercise increases sympathetic activity and activates the LC; activated DRN-projecting LC neurons modulate DRN neuronal activity and can increase intracellular Ca2+ through Gq-coupled α1b adrenergic receptors.

Increases in intracellular Ca2+ can activate downstream signals that can inhibit 5' repressor element-1 under dual repression binding protein (Freud-1), a protein with reportedly potent control over 5-HT1AR gene expression (Albert et al., 2011). This protein interacts with the 5' repressor element site (FRE), located upstream from the 5-HT1A promoter, to repress 5-HT1AR gene expression. It's possible that exercise-induced activation of the LC-DRN circuit can de-repress 5-HT1AR gene expression; however, the molecular mechanisms underlying how early life exercise could alter this circuit to produce long-lasting increases in 5-HT1AR is unknown.

The PFC of interest because it is a robust modulator of DRN activity during stress. Glutaminergic pyramidal neurons within certain parts of the medial prefrontal cortex send descending projections that synapse onto GABAergic interneurons, which in turn, inhibit stress reactive DRN 5-HT neurons. Indeed, activation of this circuit during IS prevents the behavioral, neurobiological and physiological consequences of IS (reviewed in (Maier & Watkins, 2010)). However, Greenwood et al (2013) demonstrated that this circuit is not involved in the stress protective effects of adult exercise, as PFC lesions did not prevent the development of exercise-induced stress resistance. Yet, the role of this circuit in the long lasting stress resistance produced by early life exercise remains unexplored. Indeed, the PFC undergoes significant development and restructuring throughout the juvenile and adolescent periods, and early life-exercise could capitalize upon this sensitive period and produce lasting plasticity within this circuit that is later capable of inhibiting DRN activity during IS.

Research also suggests that perturbing the microbiota during the juvenile period can lead to changes in behavior, demonstrating a link between gut microbes and the brain during this particular developmentally sensitive time. Interestingly, we have recently demonstrated that six weeks of exercise initiated during the juvenile period produces unique changes in gut microbial

organisms (Mika & Fleshner, 2016; Mika et al., 2015b). Specifically, early life exercise is capable of increasing probiotic bacterial species that have been implicated in promoting psychological health. It is possible that microbial signals from early life exercise-enhanced bacterial species may promote long-lasting changes in stress responsive neurocircuitry during this developmentally receptive time, and together with other mechanisms, interact with the developing brain to promote stress resistance.

Taken together, these data demonstrate that positive experiences in early stages of development, like exercise, can produce adaptive changes in behavior and brain plasticity that persist. These results are broadly translational to humans, as exercise at younger ages could counteract negative effects from stressful of traumatic events experienced later in life. Furthermore, this work and future work along these lines could inform policies concerning the importance of physical activity programs for children.

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CHAPTER II

EARLY LIFE EXERCISE MAY PROMOTE LASTING BRAIN AND METABOLIC HEALTH THROUGH GUT BACTERIAL METABOLITES

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i. Abstract

The 100 trillion microorganisms residing within our intestines contribute roughly 5 million additional genes to our genetic gestalt, thus posing the potential to influence many aspects of our physiology. Microbial colonization of the gut shortly after birth is vital for the proper development of immune, neural and metabolic systems, while sustaining a balanced, diverse gut flora populated with beneficial bacteria is necessary for maintaining optimal function of these systems. Although symbiotic host-microbial interactions are important throughout the lifespan, these interactions can have greater and longer lasting impacts during certain critical developmental periods. A better understanding of these sensitive periods is necessary to improve the impact and effectiveness of health promoting interventions that target the microbial ecosystem. We have recently reported that exercise initiated in early life increases gut bacterial species involved in promoting psychological and metabolic health. In this review, we emphasize the ability of exercise during this developmentally receptive time to promote optimal brain and metabolic function across the lifespan through microbial signals.

ii. Introduction

a. The gut microbiota in early life: a window of opportunity for better health

A rapidly growing body of experimental evidence has advanced our understanding of the developmental trajectory of the gut microbiota. Following the initial determining events such as delivery mode, diet, and antibiotic exposure, adult-like microbial communities gradually emerge 1-3 years after birth (Backhed et al., 2015; Yatsunenko et al., 2012); yet the young gut remains considerably different from the adult gut throughout childhood (Ringel-Kulka et al., 2013; Yatsunenko et al., 2012) and adolescence (Agans et al., 2011). Studies have demonstrated that microbial diversity progressively increases with age (Yatsunenko et al., 2012), and the microbial ecosystem of children (Ringel-Kulka et al., 2013) and juvenile rats (Mika et al., 2015) is more volatile as well as less stable and diverse than adults. The volatility and lack of diversity characteristic of an immature gut suggest that it may more sensitive to environmental forces (Ringel-Kulka et al., 2013), while the increase in complexity over time imparts stability and makes the adult gut less receptive to change. Although the mature microbiota remains dynamic and responsive to internal and external influences such as diet and diurnal rhythms (Thaiss et al., 2014) throughout the lifespan, the young gut may be comparatively more malleable before establishing a stable adult profile. These findings suggest that there are developmental periods during which lifestyle and environmental factors can leave a substantial imprint on gut microbial community structure.

The state of the microbial ecosystem during childhood and adolescent development is of particular interest because of the tremendous physical growth and neurobiological and behavioral maturation occurring at this time. Throughout this period, the developing physiological systems of the host are also uniquely plastic and receptive to modification by both internal and external

signals. This concept of developmental programming, meaning that a discrete event earlier in life can have considerable impact on the organism later in life, has been extensively considered with regard to events occurring during perinatal development. During early infant development, the gut microbiota is capable of producing significant changes in function that persist throughout life; initial colonization and subsequent early relations between the gut and the host during the postnatal period program immune, neural and metabolic systems. However, the organism may remain receptive to programming events throughout childhood and adolescent development, a period during which substantial physical, social, and psychological maturation occurs (Spear, 2000). Although host-microbiota interactions during childhood and adolescent development have been significantly understudied, there are a few studies that suggest continuous interactions between the gut microbiota and the host during childhood and adolescence can shape metabolic pathways (Cho et al., 2012), brain plasticity and behavior (Sudo et al., 2004). If the microbiota can continue to impact central and peripheral physiological systems throughout this time, childhood and adolescent development represents a promising and previously unexplored window of opportunity for health-enhancing microbiota to produce lasting adaptations in host physiology.

Childhood and adolescent developmental periods are uniquely sensitive because of the plastic state of the immature microbial ecosystem and the receptive nature of the developing host to microbial signals. Thus, treatments or interventions aiming to produce positive outcomes by targeting the microbiota are more likely to achieve persistent, favorable changes in both microbial ecology and host health if they are implemented during this window of opportunity. We highlight exercise in early life as a powerful, feasible treatment that can increase beneficial bacterial species involved in enhancing metabolic function and mood, and potentially create

lasting adaptations in lean mass and psychological wellbeing. Host brain and metabolic development are discussed in more detail below, with an emphasis on how exercise during this period can promote optimal development of these systems by promoting health-enhancing microbial species.

iii. Exercise and the gut microbiota

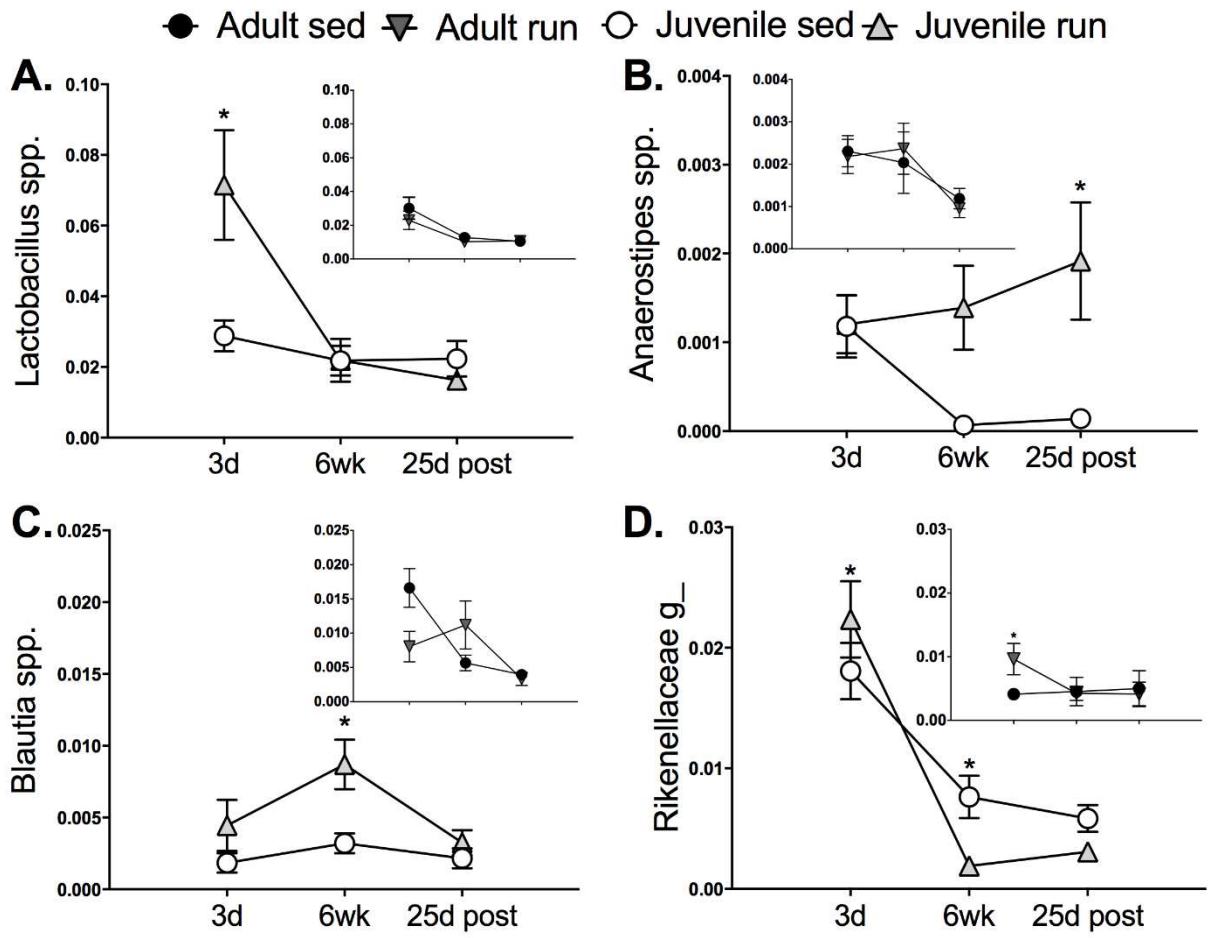
a. Exercise: an emerging modulator of health-promoting microbes

Exercise can adaptively alter the gut microbiota. Several recent studies, for example, have demonstrated that exercise can modulate the gut flora in a manner that benefits health (Clarke et al., 2014b; Evans et al., 2014; Kang et al., 2014; Matsumoto et al., 2008; Petriz et al., 2014; Queipo-Ortuno et al., 2013; Santacruz et al., 2009). While exercise confers countless favorable health outcomes that may be independent of host-microbiota interactions, there is evidence to suggest that some of these effects may be mediated by exercise-induced adaptations in gut microorganisms. One of the first studies to offer support for this idea demonstrated that an exercise-altered microbiota can promote the growth of butyrate-producing bacteria (Matsumoto et al., 2008). Butyrate is a short chain fatty acid (SCFA) that produces far-reaching adaptations in immune function (Furusawa et al., 2013), metabolism (i.e., inhibits fat accumulation and promotes leanness (Gao et al., 2009)), as well as brain plasticity and function (Intlekofer et al., 2013). Exercise can also increase gut *Lactobacillus* spp. (Queipo-Ortuno et al., 2013), a probiotic bacteria with extensively documented beneficial behavioral (Bravo et al., 2011; Messaoudi et al., 2011; Rao et al., 2009) and metabolic (Aronsson et al., 2010; Drissi et al., 2014) effects. Thus, among its many other beneficial effects, exercise can produce gut microbial changes capable of favorably impacting host health.

b. Altering the gut through early life exercise to promote lifelong health

Interestingly, we have recently demonstrated that exercise initiated in the juvenile period (immediately post weaning, analogous to childhood in humans) has a greater impact on the gut microbiota than exercise initiated in adulthood (Mika et al., 2015). Exercise begun earlier in life shifted the microbial ecosystem at the phyla level by increasing Bacteroidetes and decreasing Firmicutes, a pattern associated with a lean body type. In addition, early life exercise increased bacterial genera that are capable of producing butyrate, influencing metabolic pathways involved in fat accumulation, building and maintaining lean mass, and regulating emotional behavior (Figure 1). Specifically, early life exercise significantly increased two genera, *Blautia* spp. and *Anaerostipes* spp., within the butyrate producing family Lachnospiraceae (Vital et al., 2014). In addition, genera within the Rikenellaceae family were modulated by early life exercise, and bacteria within this family are also capable of producing butyrate (Vital et al., 2014). Probiotic Lactobacillus spp. were also increased following just 3 days of exercise. Importantly, adult onset exercise did impact the gut microbiota (Figure 1 inset), albeit to a modest degree and in a more restricted fashion (genus but not phyla-level changes). We thus provide compelling evidence that the young gut microbiota is more sensitive to exercise-induced changes in beneficial bacteria.

Figure 1



Patterns in *Lactobacillus* spp. and potential butyrate-producing genera, identified using 16S rRNA gene sequencing. Patterns in *Lactobacillus* spp. and potential butyrate-producing genera, identified using 16S rRNA gene sequencing (Mika et al., 2015), for adult and juvenile runner and sedentary rats following 3 days (3d) and 6 weeks (6 wk) of exercise, and 25 days after the end of exercise (25d post). Relative abundance of *Lactobacillus* spp. decreased across time ($p<0.005$), and juveniles exhibited more *Lactobacillus* spp. overall ($p<0.001$). A run-by-age interaction ($p<0.05$) and subsequent post hoc analyses revealed that *Lactobacillus* spp. were significantly increased 3 days following exercise onset (a). Juvenile onset exercise overall increased relative abundance of *Anaerostipes* spp. ($p<0.05$) and Blautia spp. ($p<0.05$). Following a run-by-age interaction in both genera ($p<0.05$), post hoc analyses revealed that *Anaerostipes* spp. were significantly increased in juvenile runners 25 days after exercise ended (b), whereas *Blautia* spp. were increased following 6 weeks of exercise (c). No patterns within the adult groups were found within these genera (a–c, insets). Juvenile onset exercise overall decreased a species within the Rikenellaceae family ($p<0.05$), and a run-by-age interaction ($p<0.05$) revealed that in the juvenile runners, it significantly increased following 3 days of exercise, then decreased after 6 weeks (d). Adult onset exercise (d, inset) also increased the relative abundance of this species after 3 days of exercise. Data are represented as mean ± s.e.m., *$p<0.05$.

Exercise initiated earlier in life also may produce a greater impact on the developing host. Exercise is a powerful means of improving health throughout the lifespan, and the widespread metabolic (Booth et al., 2012) and neural adaptations (Greenwood & Fleshner, 2011) produced by exercise training are extensive and well documented at all ages. However, there is a small but convincing literature demonstrating that exercise initiated during early, critical periods can produce persistent adaptations in body composition (Patterson et al., 2008; Shindo et al., 2014), brain plasticity and behavior (Hopkins et al., 2011; McKercher et al., 2014). This evidence thus suggests that exercise initiated during sensitive developmental periods can produce more robust and longer lasting health benefits, though the mechanisms by which this occurs are not fully understood. Given that early life exercise increases bacterial species capable of interacting with brain and metabolic development, these bacterial species could be mechanistically involved in some of the persistent health promoting effects of exercise.

iv. Early life exercise, microbiota, and the developing brain

a. Childhood and adolescent brain development

Childhood and adolescent brain development is distinguished by a major wave of plasticity during which axons and synapses are rapidly overproduced and then eliminated during subsequent pruning and restructuring events. Major circuits and neurotransmitter systems within cortical and limbic regions underlying these behaviors undergo substantial re-organization during this time, allowing for the maturation of higher order brain functions, cognition, social behaviors, emotion regulation, and stressor reactivity (Spear, 2000). Recent longitudinal human neuroimaging studies have confirmed that grey and white matter within cortical and limbic regions such as the prefrontal cortex, amygdala, and hippocampus peak at slightly different times within this period (Giedd et al., 2015). Rodent studies have also demonstrated re-organization of

several key neurotransmitter systems. For example, age-dependent changes within the gamma-aminobutyric acid (GABA) and serotonin (5-HT) systems within cortical and limbic regions are evident throughout this time (Morilak & Ciaranello, 1993; Z. Y. Yu et al., 2006). The GABAergic system is the major source of inhibitory neurotransmission within the brain, and among its many functions, serotonin is important for regulating mood and emotion. These studies collectively illustrate that cortical and limbic circuits and neurotransmitters systems undergo dramatic alterations during childhood and adolescence, creating a period of heightened sensitivity to internal and external signals. While disruptions in the development of these circuits can lead to psychiatric disorders, perhaps promoting neuroplasticity throughout this time may promote higher cognitive functioning and greater resistance to challenges that threaten emotional wellbeing.

b. The effects of exercise on childhood and adolescent brain development

Exercise initiated in childhood can promote brain function. For instance, exercise during the juvenile period produced persistent increases in brain-derived neurotrophic factor (BDNF) and complementary improvements in memory function when examined in adulthood (Hopkins et al., 2011). Furthermore, human and rodent studies suggest that exercise initiated during childhood and adolescence can protect the brain against stress-induced psychiatric disorders, such as depression and anxiety. For instance, rodent studies demonstrate that exercise in early life can reverse the lasting deleterious effects of early life stress on brain and behavior, including depressive-like behavior (for review, see (Harrison & Baune, 2014)). In addition, an epidemiological human study demonstrated that individuals who were habitually active since childhood were significantly less likely to develop depression in adulthood compared to their inactive counterparts (McKercher et al., 2014). Despite these notable findings that the brain can

benefit from health promoting lifestyle factors such as exercise, the positive effects of adaptive manipulations like exercise during sensitive developmental periods are understudied.

c. Microbial modulation of brain and behavior

Research within the last few decades has convincingly demonstrated that the gut microbiota can impact behavior. Germ-free (GF) mice models, or mice born in sterile conditions devoid of a microbial ecosystem, exhibit altered brain function and behavior (G. Clarke et al., 2013; Diaz Heijtz et al., 2011; Stilling et al., 2015). Notably, one study illustrated a clear link between behavior and gut bacteria by showing that colonization of GF, non-anxious mouse strains with microbial populations from anxiety-prone mice produced anxious phenotypes and vice-versa, microbiota from non-anxious strains produced "calm" phenotypes (Bercik et al., 2011). Furthermore, specific species can be administered to rodents and humans to produce adaptive behavioral and emotional effects. For example, *Lactobacillus* spp. can positively impact mood and stressor reactivity, and produce alterations in limbic circuits underlying these behaviors. In rats, *Lactobacillus rhamnosus* decreased depressive-like/anxiety-like behavior, attenuated hypothalamic-pituitary-adrenal (HPA)-axis activation following a stress challenge, and produced region-specific changes in GABA receptor sub units (Bravo et al., 2011). In both rats and humans, administration of a probiotic formula including *Lactobacillus helveticus* reduced anxiety (Messaoudi et al., 2011). In humans, *Lactobacillus casei* decreased symptoms of anxiety in patients suffering from chronic fatigue syndrome (Rao et al., 2009). Interestingly, recent studies demonstrated that prebiotics, non-digestible complex carbohydrates that are fermented into SCFAs within the colon, attenuate stress-induced anxiety (Tarr et al., 2015). Prebiotics can influence behavior by promoting the growth of beneficial bacterial species such as *Lactobacillus*, or by increasing SCFAs such as butyrate, which can directly produce

antidepressant-like effects (Schroeder et al., 2007). Collectively, these findings reveal that microbiota as well as microbial metabolites can impact behavior and brain plasticity.

d. Microbial modulation of brain and behavior may be age-dependent

The brain is sensitive to microbial signals in early life. One of the first studies to demonstrate that microbial presence during childhood can have enduring impacts on brain function showed that GF mice exhibit exaggerated HPA responses to stress, which were partially normalized following colonization with bacteria from normal mice only if colonization occurred at 6 weeks, but not at 8 weeks of life (Sudo et al., 2004). Since then, other studies have investigated how microbial composition during childhood can impact brain function by studying the effects of colonization immediately post weaning. Notably, Clarke et al (2013) demonstrated that post-weaning colonization was able to successfully restore alterations in anxiety-like behavior, but not increased levels of hippocampal 5-HT and 5-HT metabolites (G. Clarke et al., 2013). Recently, analysis of gene expression within the amygdala of GF mice revealed patterns suggestive of a hyperactive amygdala; post-weaning colonization attenuated some, but not all, of these patterns (Stilling et al., 2015). These results provide preliminary support for the idea that microbial influence throughout childhood can produce enduring changes in some discrete brain functions. More research is needed to further untangle what developmental periods are important for shaping certain behaviors through host-microbial interactions.

e. How gut microbes can influence the brain: mechanisms

Research has only begun to reveal the mechanisms behind the complex interactions of how microbiota-derived signals communicate with the brain. There is evidence that gut microorganisms can signal the brain through the vagus nerve (Forsythe et al., 2014). Various metabolites and signaling molecules that are produced by bacteria, such as SCFAs,

neurotransmitters and cytokines from immune cells, can activate adjacent receptors on vagal afferents of the enteric nervous system. Subsequently, the nucleus of the solitary tract (NTS) receives microbiota-derived vagal signals and further propagates these signals to various projection regions, including limbic structures important for mood and behavior. Several studies support the role of the vagus nerve as a key communication relay between the gut microbiota and the brain. For instance, inactivating the vagus nerve prevents several reported neurobiological effects of Lactobacillus spp. (Bravo et al., 2011).

Gut microbiota to brain signaling likely also occurs independently of the vagus; microbes can produce neurotransmitters and neurotransmitter-like substances, or alter metabolic pathways involved in neurotransmitter synthesis. For instance, *Lactobacillus* spp. are capable of producing GABA as well as serotonin (Clarke et al., 2014a). SCFA production is another means by which gut microorganisms can communicate with the brain. The SCFA butyrate in particular has well documented neurological effects. In addition to signaling the brain via the vagus nerve, it can interact with brain function through a variety of other pathways. Notably, butyrate is a histone deacetylase inhibitor and, given that it is a hydrophobic molecule, can cross the blood-brain barrier. Intraperitoneal butyrate administration can increase BDNF promoter acetylation, enhance BDNF transcription within the hippocampus, and promote hippocampal-dependent learning (Intlekofer et al., 2013) as well as produce antidepressant-like effects (Schroeder et al., 2007). Butyrate administration can also increase mRNA and protein for the transcriptional co-activator peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α; Gao et al., 2009). PGC-1α activation of the PGC-1α-PPARα/δ pathway can increase expression of kynurenine aminotransferases within skeletal muscle. These enzymes are capable of converting kynurenine into kynurenic acid and thus preventing kynurenine from crossing the blood brain barrier. This

change in kynurenine metabolism prevented stress-induced depressive-like behavior (Agudelo et al., 2014). More indirectly, both *Lactobacillus* spp. and butyrate exert anti-inflammatory effects by promoting intestinal barrier integrity (Brahe et al., 2013; Q. Yu et al., 2015). Increased bacterial translocation has been previously associated with depressive symptoms (Maes et al., 2012), thus *Lactobacillus* spp. and butyrate may protect against or attenuate such symptoms by preventing translocation of bacteria from the intestines. Though these complex interactions are not fully understood, these recent studies demonstrate that certain microorganisms produce enzymes and signaling molecules that can interact with the brain through diverse pathways.

v. Early life exercise, microbiota, and the developing body

a. Childhood and adolescent metabolic development

Although less discrete than brain developmental periods, metabolic development is also characterized by rapid maturation and is sensitive to long-term programming by external factors. This is evident during fetal and infant development. At this time, the developing organism is sensitive to perinatal metabolic programming, where the prenatal metabolic and endocrine state and the postnatal nutritional state set the stage for metabolic development and function throughout the lifespan. For instance, maternal obesity is associated with body weight patterns of offspring (Oken et al., 2008). Similarly, postnatal caloric intake is associated with long lasting changes in body weight, adiposity, and cardiac function (Velkoska et al., 2008). Given that childhood and adolescence is characterized by a comparable period of dramatic physical growth and physiological change, environmental and lifestyle factors at this time can also influence rapid metabolic development and help determine health outcomes in adulthood. Evidence for this notion is supported by results from studies demonstrating that cardiovascular and metabolic diseases originate in childhood, including those reported in the Cardiovascular Risk in Young

Finns study. This study followed a large cohort of subjects from early childhood to adulthood in order to assess cardiovascular risk factors originating in early life. Recent results demonstrate that dietary fatty acids in childhood are associated with blood pressure in adulthood (Kaikkonen et al., 2012). Similarly, research has established a connection between childhood overweight status and obesity later in life. Indeed, it was recently demonstrated that obesity in older children significantly increased risk for adult obesity (Whitaker et al., 1997). Collectively, these studies demonstrate that lifestyle and environmental factors during developmentally sensitive times can impact later life health outcomes. In particular, poor diet and BMI during childhood can dramatically affect adult metabolic health.

b. The effects of exercise on childhood and adolescent metabolic development

Health promoting lifestyle choices such as exercise during critical developmental windows can produce positive health outcomes later in life. Both human and animal studies illustrate the ability of exercise to produce persistent beneficial physiological effects. Human studies show that childhood exercise can decrease adiposity as well as risk factors for cardiovascular and metabolic disease and improve muscular and skeletal health (Ortega et al., 2008). Rodent studies have demonstrated that a transient bout of exercise initiated in juvenile rats produces long lasting adaptations in body composition in rats predisposed to obesity (Patterson et al., 2008; Shindo et al., 2014). Notably, in a rat model of obesity, prepubertal exercise decreased body weight gain and increased lean mass throughout adolescence and into adulthood, even after exercise had stopped (Shindo et al., 2014). This lasting increase in lean mass was paralleled by lasting increases in proteins known to adaptively regulate energy expenditure, including citrate synthase, and uncoupling protein-3 in the skeletal muscle. An increase in lean mass produced by early life exercise also occurs in non-obese rats. In a recent

study, we reported that exercise initiated in the juvenile period produced persistent increases in lean mass (Mika et al., 2015). Thus, exercise initiated prior to adulthood can lead to a sustained inhibition of body fat mass weight gain and persistent adaptations in skeletal muscle mass, in both obese predisposed and lean organisms. This evidence from rodent and human studies supports the notion that exercise during childhood can improve metabolic health throughout the lifespan.

c. Microbial modulation of host metabolism

There is a growing body of literature supporting a relationship between microorganisms and metabolic processes; this work has demonstrated that microorganisms regulate host energy harvest. For example, GF mice exhibit decreased body fat (Backhed et al., 2004), intestinal SCFA levels, and increased excretion of calories compared to conventionally raised mice (Tremaroli & Backhed, 2012). While the overall presence of an intact gut may increase energy harvest, the composition of the gut ecosystem is an important regulator of these metabolic processes as different bacterial species exhibit different metabolic capabilities. Broad community patterns at the phylum level can be discerned between lean and obese organisms, with a large majority of studies showing elevations in Bacteroidetes and decreases in Firmicutes in lean organisms and vice versa for obese organisms (Ridaura et al., 2013; Tremaroli & Backhed, 2012). Furthermore, altering an organism's gut bacterial composition can alter their body composition. An elegant study demonstrated that GF mice inoculated with microbiota from lean and obese humans either remained lean or gained weight, matching the phenotype of their donors. This study also demonstrated that the microbiota of lean organisms produces greater amounts of SCFAs, including propionate, acetate and butyrate (Ridaura et al., 2013). In addition to serving as a source of energy for the host, SCFAs have extensive adaptive effects on host

energy metabolism (Gao et al., 2009). Recent work also demonstrates that certain probiotic species can adaptively modulate discrete metabolic pathways. Specific *Lactobacillus* spp., for example, can inhibit fat accumulation by interacting with a variety of pathways (Aronsson et al., 2010; Drissi et al., 2014). These results help strengthen the link between gut microbial composition and host metabolic processes.

d. Microbial disruptions at young ages may have lasting metabolic consequences

The composition of gut bacteria during certain critical periods in development can influence metabolic parameters in a long-lasting manner. Bacterial composition during infancy, for example, was associated with later obesity during childhood (Kalliomaki et al., 2008), while others have shown that infant antibiotic exposure was associated with greater fat accumulation in childhood (Ajslev et al., 2011; Murphy et al., 2014). In rodents, low dose antibiotics produced alterations in metabolism and body composition across the lifespan (Cox et al., 2014). Interestingly, a recent rodent study demonstrated that sub-therapeutic antibiotic use initiated during the juvenile period changed the gut microbiota, increased adiposity and altered lipid and cholesterol metabolism by the liver (Cho et al., 2012). These studies demonstrate the potential persistent metabolic consequences of disrupting microbial homeostasis during sensitive developmental periods. Fluctuations in gut microbiota may also alter body composition regardless of age; however, if metabolism-regulating host-bacteria interactions occur earlier in life, the consequences could be more robust and longer lasting. It is important to point out that these studies are investigating the lasting metabolic consequences of microbial *perturbations*. It is possible that lifestyle and environmental factors that adaptively modulate the microbiota during developmentally sensitive times promote metabolic health.

e. How gut microbes can modulate metabolism and body composition: mechanisms

Although complex interactions between microbial signals and host metabolic pathways are not fully elucidated, research within recent years has greatly improved our understanding. In addition to the impacts of changes in broad phyla level microbial patterns (i.e, low Bacteroidetes and high Firmicutes), metabolic processes are also modulated by changes in specific gut bacterial species and even strains. For instance, strain level differences in *Lactobacillus* spp. within the proximal colon can modulate carbohydrate metabolism. Certain strains are associated with protecting against weight gain, while other stains promote weight gain. Protective Lactobacillus spp. harbor enzymes that are associated with degrading fructose, dextrin and acetate, which can inhibit fat accumulation and regulate blood glucose (Drissi et al., 2014). In addition, *Lactobacillus paracasei* can decrease body fat by increasing circulating levels of angiopoietin-like protein 4 or ANGPTL4 and thus inhibiting lipoprotein lipase activity (Aronsson et al., 2010). Thus, probiotic species can adaptively regulate discrete metabolic pathways.

Bacteria may also impact host metabolism via production of SCFAs (i.e., acetate, butyrate, propionate). SCFAs decrease adiposity and enhance energy expenditure and satiety signals by interacting with multiple metabolic pathways (Tremaroli & Backhed, 2012). Notably, butyrate has received attention of its ability to produce adaptations within adipose tissue as well as skeletal muscle. For instance, butyrate can modulate triglyceride uptake with lipoprotein lipase (Korecka et al., 2013). Furthermore, butyrate can prevent obesity, improve sensitivity to insulin, produce adaptive thermogenesis, and induce various plasticity promoting pathways within skeletal muscle (Brahe et al., 2013; Gao et al., 2009). More specifically, by increasing PGC-1α mRNA and protein within the muscle, butyrate itself can initiate pathways that produce some of the same muscular adaptations as endurance exercise. For instance, in mice, butyrate-induced increases in PGC-1α increased mitochondrial biogenesis within skeletal muscle (Gao et

al., 2009). These pathways can provide resistance against muscle atrophy, thus providing a potential mechanism for the persistent increases in lean mass we observed in young runners (Mika et al., 2015). Butyrate producing bacteria may be therefore capable of enhancing lean mass and reducing fat by initiating some of the same metabolic pathways as exercise.

vi. A microbial ecosystem altered by early life exercise can potentially promote lifelong metabolic and psychological health

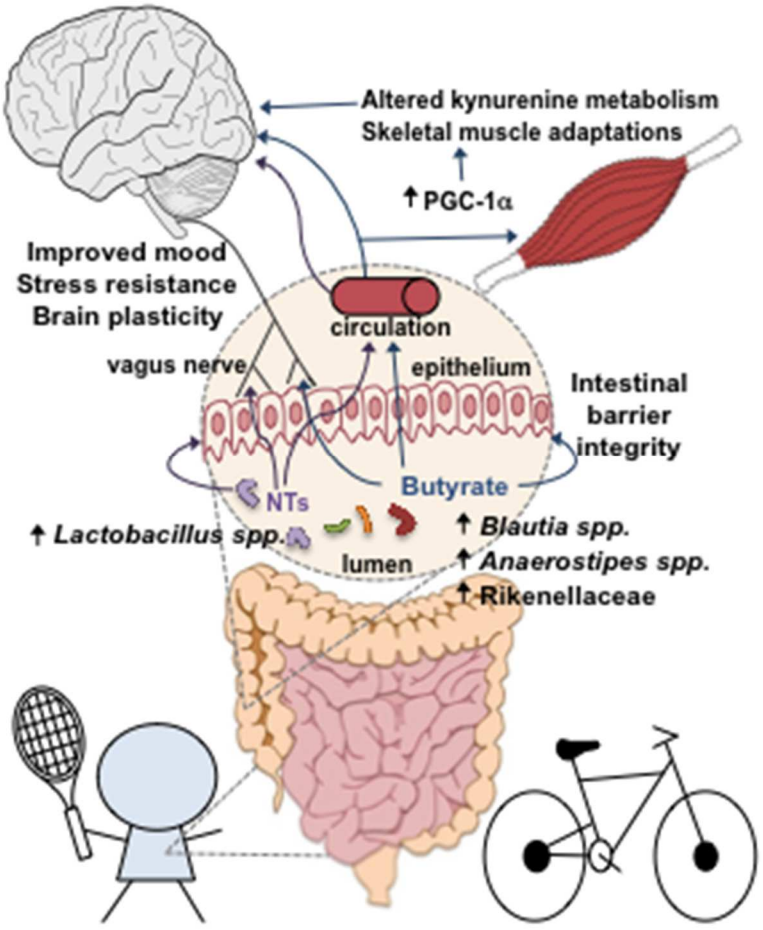
a. Early life exercise enhances multi-tasking microbiota

We have reported that exercise initiated during the juvenile period has a greater impact on the gut microbiota compared with exercise initiated in adulthood (Mika et al., 2015), and can increase probiotic *Lactobacillus* spp. as well as butyrate-producing bacteria (Figure 1). Both *Lactobacillus* spp. and butyrate producing bacteria are multi-tasking microbiota in that they interact with a variety of pathways to produce extensive neural and metabolic benefits. Both *Lactobacillus* spp. and butyrate can promote intestinal barrier integrity and prevent inflammation associated with bacterial translocation. Notably, *Lactobacillus* spp. can reduce anxiety and depressive-like behavior, alter brain chemistry, and inhibit fat accumulation. In addition, potential butyrate-induced increases in PGC-1α can potentially activate extensive signaling pathways to enhance BDNF within the brain, protect against the deleterious behavioral consequences of stress, promote skeletal muscle adaptations, and decrease adiposity. It is possible that increases in these bacteria are mechanistically involved in the ability of early life exercise to promote brain function or emotional wellbeing across the lifespan (McKercher et al., 2014), or promote persistent increases in lean mass (Mika et al., 2015; Figure 2).

Exercise may be able to achieve these benefits independently of the gut microbiota. Indeed, exercise initiated in adulthood produces many of these benefits and fewer of these

microbial changes. However, *early life* exercise can recruit the microbial system, and perhaps produce a more robust, synergistic physiological response aimed at improving health during this window of opportunity.

Figure 2



Early-life exercise increases probiotic *Lactobacillus* spp. as well as butyrate-producing species. Early-life exercise increases probiotic *Lactobacillus* spp. as well as butyrate-producing species, and these bacteria can help promote the persistent exercise-induced adaptations in brain plasticity, mood/behavior and skeletal muscle by influencing various aspects of host function. *Lactobacillus* spp. can influence mood and alter brain chemistry by producing various neurotransmitters (NTs) such as serotonin and GABA, which can activate vagal afferents or interact with circumventricular organs through the circulation. Butyrate-producing bacteria can increase butyrate, which can interact either with vagal afferents, or enter the circulation and cross the blood–brain barrier to influence behavior and brain plasticity. Butyrate can also increase the transcriptional co-activator PGC-1α within skeletal muscle, leading to adaptations in skeletal muscle or alterations in kynurenine metabolism, which can protect against stress-induced depressive-like behavior. Both *Lactobacillus* spp. and butyrate can promote intestinal barrier integrity and prevent inflammation associated with bacterial translocation through interactions with tight junction proteins or by inhibiting epithelial cell apoptosis.

vii. Concluding thoughts

Childhood and adolescence are developmental periods of plasticity described as windows of vulnerability, as exposure to negative environmental events during this time can increase pathologies including depression and obesity. We propose that these same developmental periods also provide windows of opportunity; lifestyle factors such as exercise and nutrition throughout these periods can promote optimal brain and metabolic development across the lifespan. In addition, early life changes in gut microbiota may play a previously unrecognized role in these positive effects. We suggest that early life exercise-induced changes in mood and metabolism-enhancing bacteria are potentially mechanistically involved in the persistent health benefits of exercise throughout this sensitive time. Future work should systematically explore the age-dependent contribution of microbial metabolites to host development, as well as potential mechanisms underlying some of the health benefits of exercise.

viii. References

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CHAPTER III

EARLY LIFE DIETS WITH PREBIOTICS AND BIOACTIVE MILK FRACTIONS ATTENUATE THE IMPACT OF STRESS ON LEARNED HELPLESSNESS BEHAVIORS AND ALTER GENE EXPRESSION WITHIN NEURAL CIRCUITS IMPORTANT FOR STRESS RESISTANCE

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i. Abstract

Manipulating gut microbes may improve mental health. Prebiotics are indigestible compounds that increase the growth and activity of health- promoting microorganisms, yet few studies have examined how prebiotics affect CNS function. Using an acute inescapable stressor known to produce learned helplessness behaviors such as failure to escape and exaggerated fear, we tested whether early life supplementation of a blend of two prebiotics, galactooligosaccharide (GOS) and polydextrose (PDX), and the glycoprotein lactoferrin (LAC) would attenuate behavioral and biological responses to stress later in life. Juvenile, male F344 rats were fed diets containing either GOS and PDX alone, LAC alone, or GOS, PDX and LAC. All diets altered gut bacteria, while diets containing GOS and PDX increased *Lactobacillus* spp. After 4 weeks, rats were exposed to inescapable stress, and either immediately sacrificed for blood and tissues, or assessed for learned helplessness 24 hours later. Diets did not attenuate stress effects on spleen weight, corticosterone and blood glucose; however, all diets differentially attenuated stress-induced learned helplessness. Notably, *in situ* hybridization revealed that all diets reduced stress-evoked *cfos* mRNA in the dorsal raphe nucleus (DRN), a structure important for learned helplessness behaviors. In addition, GOS, PDX and LAC diet attenuated stress-evoked decreases in mRNA for the 5-HT1A autoreceptor in the DRN and increased basal BDNF mRNA within the prefrontal cortex. These data suggest early life diets containing prebiotics and/or LAC promote behavioral stress resistance and uniquely modulate gene expression in corresponding circuits.

ii. Introduction

Certain gut microbes can benefit mental health. Notably, *Lactobacillus* spp. can attenuate the effects of stress in rodents (Ait-Belgnaoui et al., 2014; Ait-Belgnaoui et al., 2012; Bravo et al., 2011) and improve symptoms associated with anxiety and depression in humans (Messaoudi et al., 2011; Rao et al., 2009; Steenbergen et al., 2015). Manipulations that increase probiotic microorganisms thus warrant further study as countermeasures for stress-related psychiatric disorders.

Prebiotics are dietary compounds that resist gastric enzymes and are instead fermented by endogenous bacteria capable of utilizing them as substrates (Bouhnik et al., 1997; Moro et al., 2002). The prebiotics galactooligosaccharide (GOS) and polydextrose (PDX) increase beneficial microorganisms, including Lactobacillus spp. (Cardelle-Cobas et al., 2011; Herfel et al., 2011; Saulnier et al., 2013; Schwab & Ganzle, 2011), and can synergistically enhance microbiota and health (Scalabrin et al., 2012; Ziegler et al., 2007). Similarly, the glycoprotein lactoferrin (LAC) has prebiotic (Otsuki et al., 2014), antimicrobial and microbiostatic (Alexander et al., 2012; Leon-Sicairos et al., 2006) capabilities, and may foster conditions that support beneficial bacteria (Mastromarino et al., 2014).

Some prebiotics affect the central nervous system. Formulations of GOS increased brain-derived neurotrophic factor (BDNF), plasticity molecules (Savignac et al., 2013; Williams et al., 2016), and prevented inflammation and anxiety following immune challenge (Savignac et al., 2016), while human milk derived prebiotics protected against stress-induced anxiety (Tarr et al., 2015). LAC also promoted learning and signaling pathways involved in neuroplasticity (Chen et al., 2015). Furthermore, GOS, PDX and LAC combined enhanced brain structure (Mudd et al., 2016; Waworuntu et al., 2016). Notably, prebiotics may be more effective in early life (Chen et

al., 2015; Mudd et al., 2016; Waworuntu et al., 2016; Williams et al., 2016), when the developing brain (Spear, 2000) and microbiota (Agans et al., 2011; Ringel-Kulka et al., 2013; Yatsunenko et al., 2012) are more plastic. Given that prebiotics are safe and commercially available, it is important to identify formulations capable of improving mental health.

We therefore tested whether early life supplementation of LAC and a GOS and PDX blend, alone or combined can increase *Lactobacillus* spp. and prevent the consequences of inescapable stress (IS). IS produces learned helplessness (LH), behaviors that mirror some symptoms of human depression and anxiety. Since the neural mechanisms behind IS-induced LH are established, this paradigm is useful for elucidating how novel interventions protect against stress. Briefly, the development of LH behaviors is due to hyperactivation of dorsal raphe nucleus (DRN) serotonergic (5-HT) neurons during IS (Grahn et al., 1999; Maswood et al., 1998). IS renders these neurons temporarily sensitized so that 24 hours later, behavioral testing produces excessive 5-HT release from DRN neurons projecting to sites controlling these behaviors, like the amygdala and striatum (Amat et al., 1998a, 1998b).

Interventions that protect against IS-induced LH modulate activity and gene expression in circuits controlling LH behaviors. For instance, both exercise (Greenwood et al., 2005a; Greenwood et al., 2003a; Greenwood et al., 2012a) and behavioral control (Amat et al., 2005; Grahn et al., 1999; Maier & Watkins, 2005) attenuate cfos in DRN 5-HT neurons following IS and prevent learned-helplessness behaviors 24 hours later. Exercise increases in mRNA for the autoinhibitory 5-HT1A receptor (5-HT1AR) in the DRN (Greenwood et al., 2003a) potentially buffer against DRN 5-HT neuron hyperactivation during IS, while reductions in mRNA for postsynaptic 5-HT2C receptors (5-HT2CR) in DRN projection regions (Greenwood et al., 2012b) may decrease sensitivity to 5-HT during behavioral testing. Additionally, behavioral

control constrains *cfos* mRNA within the amygdala (Weinberg et al., 2010) and in the prefrontal cortex modulates growth factor gene expression in the prefrontal cortex (PFC), which can control DRN activity during IS (Bland et al., 2007).

We examined if GOS and PDX alone, LAC alone, or GOS and PDX and LAC fed to juvenile rats could constrain the impact of IS on the stress response and LH, and modulate *cfos*, 5-HT1AR, 5-HT2CR, BDNF, and gamma-aminobutyric acid (GABA) receptor subunit mRNA within corresponding circuits in adulthood.

iii. Materials and methods

a. Subjects

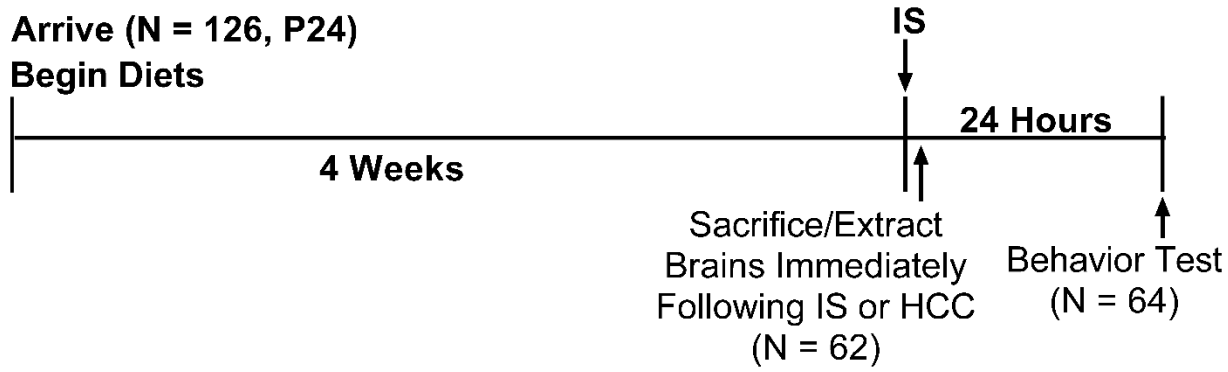
Juvenile (PND 24), male Fischer 344 rats (Envigo; Indianapolis, IN) were housed in a temperature (22°C) and humidity controlled environment and maintained on a 12:12 h light/ dark cycle (lights on from 0600-1800). The rats were pair housed in standard Nalgene Plexiglas cages (45cm × 25.2cm × 14.7cm) and had *ad libitum* access to food and water for the duration of the experimental procedures. Experimental protocols for these studies were approved by the University of Colorado Animal Care and Use Committee and care was taken to ensure minimal discomfort during all procedures.

b. Experimental design

We investigated whether diets formulated with LAC, GOS, and PDX attenuated IS-induced LH behaviors and accompanying changes in gene expression in brain regions controlling behavioral responses to stress. Rats began diets on postnatal day 24 (PND 24) because interventions produce greater adaptations within microbial ecology if begun earlier in development (Mika et al., 2015). Immediately upon arrival, rats were randomly assigned to one of the four dietary conditions (Figure 1): control diet (CON); LAC; GOS, PDX; GOS, PDX,

LAC. Food consumption was monitored three times per week by weighing the chow pellets within each food hopper. Rats were weighed and fecal pellets were collected once per week. Following 4 weeks on the diets, rats were exposed to IS or remained within their home cage undisturbed (home cage controls; HCC). Half of the rats from each diet were sacrificed immediately following IS procedures (see below), and brains and plasma were extracted for later analysis of dietary impacts on gene expression within the brain and physiological indicators of hypothalamic-pituitary adrenal (HPA) axis and sympathetic nervous system activation (n=8/grp used for brains and plasma, rats were excluded if statistical analyses revealed outliers or if brain tissue integrity precluded tissue from analysis). Changes in gene expression was investigated immediately following stress because peak *cfos* mRNA levels occur immediately-1 hour following IS (Bland et al., 2005; McDevitt et al., 2009) and similarly, stress-induced changes in BDNF mRNA are present immediately after IS (Greenwood et al., 2007). In addition, we chose this time point to maximize our potential of capturing diet-induced modulation of stress-induced changes in gene expression, as other stress-protective manipulations, such as exercise, produce the greatest modulation of gene expression within the DRN at this time (Loughridge et al., 2013). 24 hours later, the remaining rats were assessed for anxiety and depressive-like behaviors. (n=8/grp used for behavioral experiments, rats were excluded if statistical analyses revealed outliers). These behaviors were investigated 24 hours following stress because these behaviors are not detectable immediately and are strongly evident 24 hours following stress, yet are absent 72 hours following stress (reviewed in Maier and Watkins (2005).

Figure 1



Experimental timeline. Upon arrival, juvenile male Fischer 344 rats immediately began either experimental diets or control diet. Following 4 weeks on the diets, rats were exposed to IS or remained within their home cage (HCC). Half of the rats from each diet were sacrificed immediately following IS, and brains and plasma were extracted for later analysis. 24 h later, the remaining rats were assessed for anxiety and depressive-like behaviors.

c. Diet

All experimenters were blind to type of diet administered to each group. The diets were formulated by Mead Johnson Nutrition (MJN, Evansville IN) based on AIN-93G specifications to be isocaloric and have similar carbohydrate, protein, fat, vitamin and mineral levels. Differing from the control, the LAC diet had the addition of 2.6g/kg, the GOS, PDX diet had the addition of 7.0 g/kg each, and the GOS, PDX, LAC diet had the addition of 2.6g/kg (LAC), 7.0g/kg (GOS) and 7.0g/kg (PDX). Diets were produced by Envigo (Indianapolis, IN).

d. Fecal sample collection

Fecal samples were collected weekly by placing each rat in a new sterile cage to induce defecation (Restrepo & Armario, 1987). Samples were obtained with forceps sterilized with 100% ethanol before and after obtaining each sample. Samples were then placed in 1.5 mL sterile, screw cap tubes (USA Scientific, FL) on ice, then stored at -80°C for later analyses. Rats were returned to their home cage immediately after sample collection. Collections occurred during their inactive cycle at approximately 0900.

e. Microbial composition analysis using selective bacterial culture

Lactobacillus-specific culture media (modified-rhamnose-2,3,5-triphenyltetrazolium (TTC) chloride-LBS-vancomycin agar; M-RTLV-agar) was adapted from Sakai and colleagues (Sakai et al., 2010) to measure the following Lactobacillus spp. : *L. rhamnosus* and *L. casei, L. paracasei, L. plantarum, L. fermentum, L. reuteri, L. sakei.* M-RTLV agar was prepared by combining L-rhamnose (0.4 g/mL), TTC (30.0 mg/mL), vancomycin (10.0 mg/mL), and metronidazole (10.0 mg/mL) with nutrient agar.

The formulation of M-RTLV agar facilitates the selective growth of vancomycin resistant *Lactobacillus* spp. In addition, it allows for visual discrimination of *L. rhamnosus* colony

forming units (CFU) from other *Lactobacillus* spp. due to the fermentative capabilities of *L. rhamnosus*. This species is capable of producing lactic acid through the fermentation of L-rhamnose. When TTC, a salt that forms a deep red precipitate when reduced, is present within the media, Lactobacillus spp. other than *L. rhamnosus* appear as deep red, symmetrical CFUs. *L. rhamnosus* will be lighter in color because the acidic conditions produced by the lactic acid prevent TTC reduction. Based on color and shape, *L. rhamnosus* colonies were isolated, sequenced by GeneWiz, and verified in the SILVA data base. Total aerobic and total anaerobic bacteria were also cultured using nutrient agar plates and anaerobic chambers when appropriate (Difco Nutrient Agar, BD; 23g/L).

Fecal samples were prepared for plating by first homogenizing 0.2g of each sample in 2.0 mL phosphate buffered saline (PBS). Homogenates were subsequently diluted in PBS (1:5000) and plated on M-RTLV-agar. Plated samples were incubated at 37° C; *Lactobacillus* spp. and anaerobic bacteria were incubated in anaerobic conditions created by a BD GasPak EZ Anaerobe Container System Sachets and placing the indicator inside a BD GasPak EZ Large Incubation Container (33.35cm × 16.51cm × 17.145cm). The anaerobic atmosphere is created by activation of inorganic carbonate, activated carbon, ascorbic acid and water once exposed to air. After 48 of incubation, the CFUs were counted using a cell counter (Scienceware Electronic Colony Counter) and dilution corrected averages were then calculated and analyzed.

f. Inescapable Stress

IS consisted of 100, 1 mA inescapable tail shocks administered at variable intervals (average ITI of 60 s) over a period of approximately 2 hours. Shock intensity was increased from 1.0 to 1.5mA after the first 50 shocks to reduce stress-induced analgesia. Rats exposed to IS were restrained in Broome-style Plexiglas tubes (23.4 cm in length and 7.0 cm in diameter) with their

tails exposed for electrode attachment. This procedure occurred during their inactive (light) cycle from 0800 to 1000 and the rats were returned to their home cage immediately after shock session termination.

g. Sacrifice and Tissue Collection

Half of the rats from each diet, along with their respective home cage controls, were sacrificed immediately following IS using rapid decapitation, sans anesthesia. Trunk blood was immediately measured with a glucose meter, then collected in EDTA tubes, and subsequently subjected to centrifugation to isolate plasma (300x*g* at 4°C for 15 min). Spleens were removed and weighed, and brains were extracted and frozen for later analysis of stress and dietary impacts on gene expression.

h. Corticosterone ELISA

An enzyme-linked immunosorbent assay (ELISA; Arbor Assays, catalog number K014-H5) was utilized to assess plasma corticosterone, in accordance to manufacturer's instructions. In place of the steroid displacement reagent, we used heat-extraction to degrade corticosterone-binding protein. Briefly, plasma samples were diluted 1:200 in distilled water in polypropylene tubes, and placed in water bath at 65°C water bath for 1 h. Samples cooled at RT for 20 min before proceeding with subsequent steps per manufacturer's instructions. Optical densities were measured using a SpectraMax plate reader at 450nm, and analyzed using Softmax Pro software.

i. Behavioral Testing

24 hours following IS, anxiety and depressive-like learned helplessness behaviors were tested in the remaining rats. Rats were placed in shuttle boxes (50.8cm × 25.4cm × 30.48cm, Coulbourn Instruments, Whitehall, PA) and assessed for shock-elicited freezing and subsequently shuttle box escape deficits within the same testing session, following previously established protocols

(Greenwood et al., 2003a). Following placement into the shuttle boxes, rats were allowed 10 minutes of exploration within the novel environment, during which the rats were scored every 10 seconds as freezing (no movement except respiration) or not freezing. Rats then received 2 fixed ratio 1 (FR-1) foot shocks (0.1 mA, 60 s ITI). Shock termination occurred once the rat crossed from one side of the shuttle box to the other, and latencies to cross were recorded. Immediately following FR-1 administration, shock-elicited freezing behavior was observed by a blind experimenter for 20 minutes to measure conditioned fear to environmental cues associated with the shuttle box environment. During this time, rats were again scored every 10 seconds as freezing or not freezing. Following assessment of shock-elicited freezing, rats were immediately assessed for the shuttle box escape deficits. Rats received 25 fixed ratio 2 (FR-2) foot shocks (0.6 mA, 60 s ITI). Shock termination occurred when the rat passed through the shuttle box door twice, and latencies to cross were recorded. If a rat did not pass through the door twice within the allotted 30 seconds, they were given a latency score of 30 and the shock was terminated. Scoring was done by an experimenter who was blind to treatment conditions. Testing occurred during the inactive cycle from 0800 to 1200, with each session lasting approximately 1 hour.

j. In Situ Hybridization

Tissue preparation and in situ hybridization procedures followed previously established protocols (Greenwood et al., 2011; Wang et al., 2015). Briefly, rats were sacrificed via rapid decapitation and brains were extracted, frozen in isopentane with dry ice (between -20°C and -30°C for 4 minutes) and stored at -80°C. Brains were then sliced in sections at 10 μm thickness at -21°C using a cryostat (Leica Biosystems, CM1950, Nussloch, Germany). Rostral-caudal sections of the brain were collected and thaw-mounted onto Superfrost Plus slides (Fisherbrand, Pittsburg, PA). Sliced tissue sections were then stored at -80°C. The following brain regions of

interest were collected: the PFC, striatum, amygdala, hippocampus, habenula, DRN, LC, and NTS.

Prior to hybridization, sections were fixed for an hour (4% paraformaldehyde), washed 3 times in 2X sodium saline citrate (SSC), acetylated for 10 minutes (0.25% acetic anhydride containing 0.1M triethanolamine), and dehydrated in graded ethanol. Riboprobes for *cfos* (576 mer, 596-1171 coding region), 5-HT1AR (911 mer, 333-1243 coding region), 5-HT2C receptor (5-HT2CR; 556 mer, 1370-1925 coding region), α2 subunit of the ionotropic $GABA_A$ receptor ($GABA_{A\alpha2}R$; 603 mer, 1603-2205 coding region), and BDNF (750 mer; 661-1410 coding region) were transcribed with the radioactive label Uridine 5'-triphosphate UTP ([^{35}S-UTP]; Perkin-Elmer, Waltham, MA, USA). Once transcription was complete, riboprobes were mixed with 50% hybridization buffer (50% high grade formamide, 10% dextran sulfate, 3X SSC, 1X Denhardt's solution, 0.2 mg/mL yeast tRNA, and 0.05 M sodium phosphate; pH 7.4). The riboprobes and hybridization buffer solution was applied to appropriate tissue slices. Slices were then incubated overnight (55°C) in humid chambers, humidified with diluted formamide solution in H_20 (60% formamide). The following morning, the slides were washed 3 times in 2X SSC and treated with RNase A (200 μL/mL) for 1 hour to degrade any unbound RNA. The slides were then rinsed in graded concentrations of SSC, then incubated in 0.1X SSC for an hour at 65 °C, and finally dehydrated in graded ethanol. Once dry, slides were exposed to X-ray film (Biomax-MR; Eastman Kodak, Rochester, NY, USA) in light tight autoradiography cassettes. The exposure time varied by probe: *cfos* films were exposed for 1 week, 5-HT1AR for 2 weeks, 5-HT2CR and BDNF for 1 week, and $GABA_{A\alpha2}R$ subunit for 36 h. Films were then developed (Konica Minolta Medical Imaging, model SRX-101A, Grand Rapids, MI, USA) in preparation of digital capture.

k. Image Analysis for in Situ Hybridization

Following previously established protocols (Greenwood et al., 2005b; Greenwood et al., 2011), computer-assisted optical densitometry was used to digitally capture (CCD camera, model XC-77; Sony, Tokyo, Japan) and analyze mRNA levels of *cfos*, 5-HT1AR, 5-HT2CR, $GABA_{A\alpha 2}R$, and BDNF. Scion Image version 4.0 (Scion, Frederick, MD, USA) software was used to calculate relative optical density of the X-ray films of brain regions of interest. A macro in Scion Image determined signal above a set background. To set the background, a sample was taken over white matter and in that sample, signal threshold was determined by calculating the mean gray value +3.5 standard deviations. The section of interest was density sliced at this value and only pixels above this set threshold were included in the analysis. Results are expressed as signal intensity (mean signal above background) multiplied by the number of pixels above the set threshold, giving the mean integrated density of each sample. Quantifications of each subject's mean integrated density occurred between the following coordinates (Paxinos and Watson, 1998): PFC (+4.20mm to +2.76 anterior to bregma), striatum (+1.60mm to +0.20mm), amygdala (-2.56mm to -3.30), hippocampus (-2.52mm to -4.36mm posterior to bregma), habenula (-3.24mm to -3.6mm), DRN (rostral, -7.40mm to -7.64mm; mid, -7.80mm to -8.00mm; caudal, -8.30 to -8.50mm), LC (-9.60mm to -10.08mm), and NTS (-13.24mm to -14.30mm). Averages of the integrated densities from 3-4 slices per region per subject gave each subject's mean integrated density for that particular riboprobe associated with the brain region.

l. Statistical Analyses

Weekly body weight and food consumption were analyzed by a 4 X 4 repeated measures ANOVA, with diet as a between factor (CON; LAC; GOS, PDX; GOS, PDX, LAC) and week on diet as a within factor (weeks 1-4). Plasma corticosterone, blood glucose, spleen weight, and

bacterial culture results were analyzed with a 4 (diet) X stress (HCC; IS) ANOVA. Bacterial culture results were analyzed using ANOVA between diets (bacteria was sampled in rats prior to IS exposure). For shock-elicited freezing behavior, pre-shock freezing scores were averaged into 1 pre-shock score and analyzed with ANOVA. Shock-elicited freezing scores were collapsed into 10, 2-minute blocks and analyzed using repeated measures ANOVA. FR-2 escape latencies were averaged into 5 blocks of 5 trials each and analyzed with ANOVAs. Two analytical approaches were utilized to analyze gene expression data. For brain structures displaying distinct and quantifiable subregions, subregion was used as a factor in our ANOVA while for structures that did not have notable subregional specificities, subregion was not a factor in our ANOVAs.

Specific structures with subregion as a factor are the following: *cfos* and 5-HT1AR mRNA within the dorsoventral DRN were analyzed using a 3 (subregion; rostral, mid and caudal) X 4 (diet) X 2 (stress) ANOVA. *cfos* mRNA and 5-HT1AR mRNA within the lateral DRN were analyzed with a 2 (subregion; rostral, mid) X 4 (diet) X 2 (stress) ANOVA. *cfos* mRNA within the bed nucleus of the stria terminalis (BNST) was analyzed with a 3 subregions (lateral ventral + fusiform region; LV + Fu, lateral dorsal; LD, and medial) X 4 (diet) X 2 (stress) ANOVA. 5-HT2CR mRNA within the amygdala was analyzed with a 3 (lateral dorsal; LD, lateral ventral; LV, and basal; B) X 4 (diet) X 2 (stress) ANOVA, while 5-HT2CR mRNA within the striatum with a 2 (dorsomedial, dorsolateral) X 4 (diet) X 2 (stress) ANOVA. $GABA_{A\alpha2}R$ and BDNF mRNA within the hippocampus were analyzed with 4 (dentate; DG, CA1, CA2, CA3) X 4 (diet) X 2 (stress) ANOVAs. BDNF mRNA within the prefrontal cortex (PFC) was analyzed with a 3 (cingulate; CG, infralimbic; IL, prelimbic; PL) X 4 (diet) X 2 (stress) ANOVA.

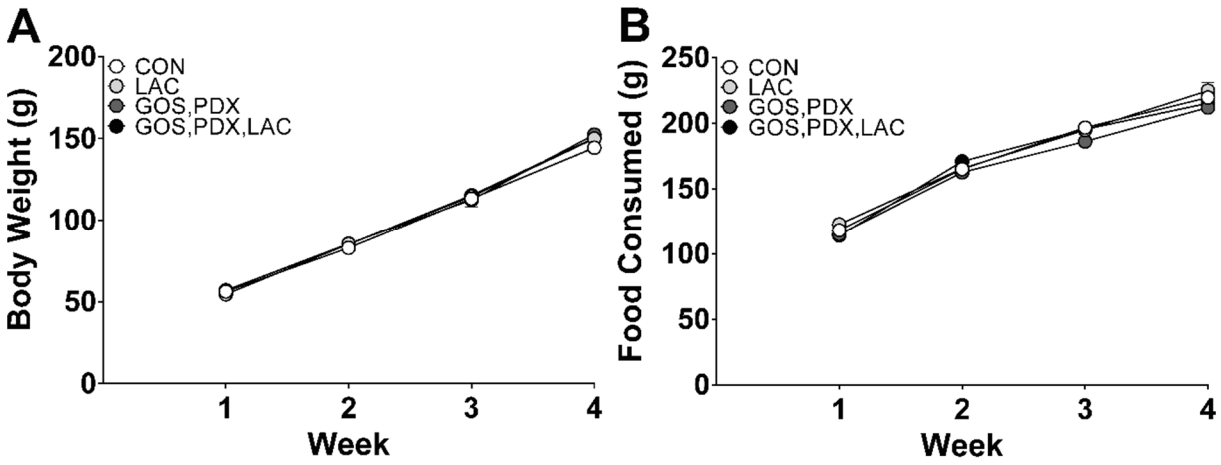
The brain regions not separated into distinct subregions during quantification include the following: *cfos* mRNA within the solitary tract nucleus (NTS), locus coeruleus (LC), habenula, amygdala and striatum, and $GABA_{A\alpha 2}R$ mRNA within the amygdala; these were all analyzed using a 4 (diet) X 2 (stress) ANOVA. Simple regressions were run on stress groups only, across all diets; regressions between bacteria and gene expression measures were run with bacteria as the independent variables and genes of interest as the dependent variables; regressions between bacteria and markers of HPA and sympathetic nervous system activation were run with bacteria as the independent variables and activity markers as the dependent variables; regressions between markers of HPA and sympathetic nervous system activation and gene expression measures were run with activity markers as the independent variables and genes of interest as the dependent variable. When necessary, post hoc analyses were performed (Fisher's PLSD). All data were screened for outliers using the appropriate outlier test (i.e., the Grubbs single outlier test or Iglewicz and Hoaglin's multiple outlier test). Significant outliers identified using these tests were excluded from analyses. Results were considered significant when $p \leq 0.05$.

iv. Results

a. No effect of diet on body weight or food consumption

All rats, regardless of diet, gained weight over the 4-week experiment ($F(3,369) = 1316.560$; $p < 0.0001$). Furthermore, diet had no effect on bodyweight ($F(3,123) = 0.282$; $p = 0.8380$; Figure 2A). Similarly, the amount of food consumed increased across four weeks ($F(3,177) = 1041.710$; $p < 0.0001$), and no differential impact of diet was found ($F(3,59) = 2.227$; $p = 0.0944$; Figure 2B).

Figure 2.

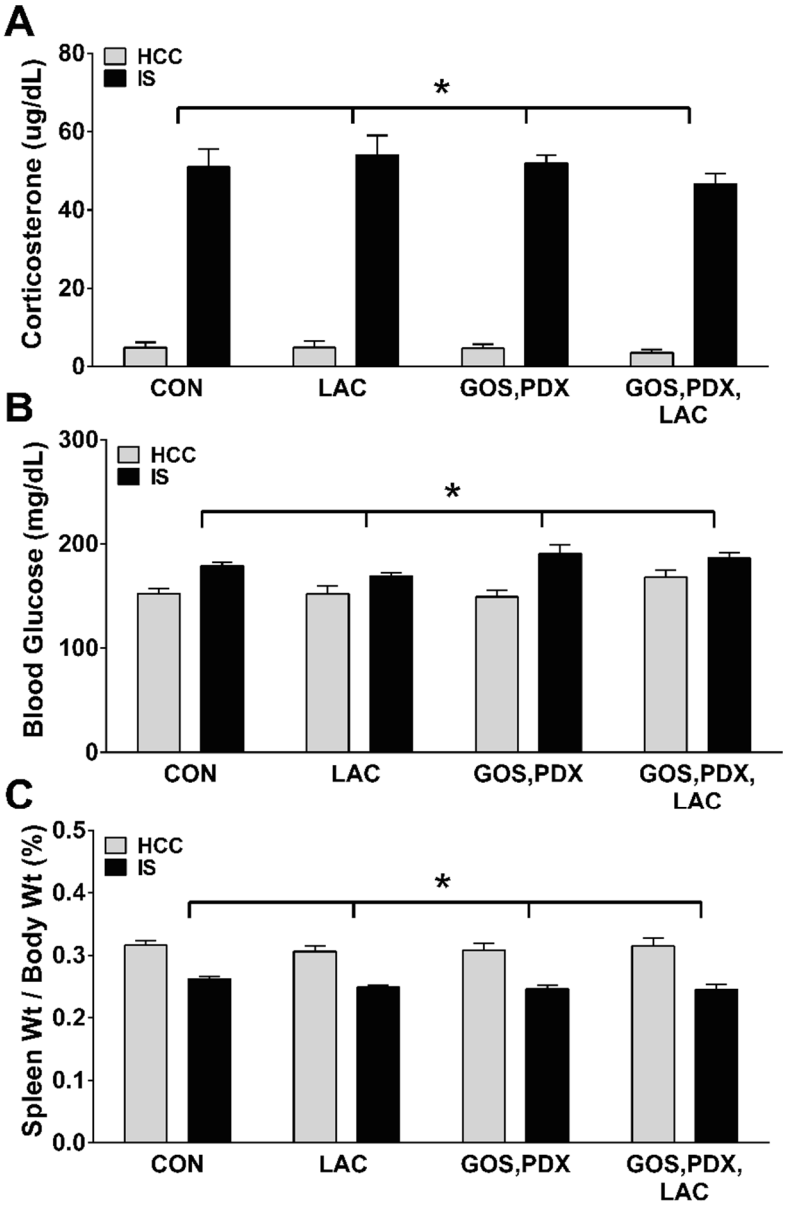


No effect of diet on body weight or food consumption. A) Body weight across four weeks of diet. B) Food consumption, measured by cage, across four weeks of diet. Data are represented as mean ± SEM. N/grp: body weight: 31-32/grp; food consumption: 15-16/grp.

b. Diet did not attenuate impact of stress on corticosterone, blood glucose, or spleen weight

IS increased corticosterone ($F(1,53) = 542.572$; $p < 0.0001$; Figure 3A) and blood glucose ($F(1,53) = 36.169$; $p < 0.0001$; Figure 3B) regardless of diet. Similarly, IS decreased spleen weight, a proxy for sympathetic nervous system activation ($F(1,53) = 109.491$; $p < 0.0001$; Figure 3C), regardless of diet.

Figure 3.

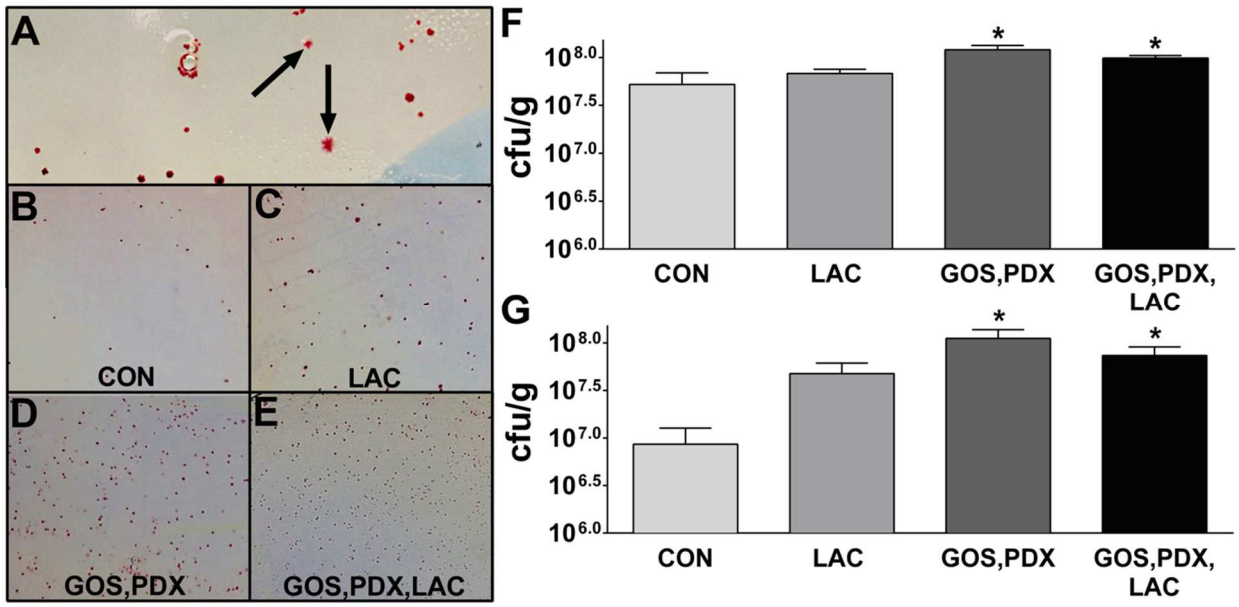


Diet did not attenuate impact of stress on corticosterone, blood glucose, and spleen weight. A) corticosterone B) blood glucose and C) spleen weight following IS, in each diet. Data are represented as mean ± SEM. * $p < 0.05$ indicates a main effect of stress. N: 7-8/grp.

c. Diet increased L. rhamnosus and L. casei, L. paracasei, L. plantarum, L. fermentum, L. reuteri, L. Sakei and impacted aerobic and anaerobic bacteria

Figures 4F and 4G depict colony forming units (cfu) of *L. rhamnosus* and other *Lactobacillus spp. (L. casei, L. paracasei, L. plantarum, L. fermentum, L. reuteri, L. sakei)*, respectively. Diet increased *L. rhamnosus* ($F(3, 26) = 6.731$; $p = 0.0016$; Figure 4F), and post hoc comparisons revealed that GOS, PDX ($p = 0.0004$) and GOS, PDX, LAC ($p = 0.0183$) diets only increased *L. rhamnosus* cfu's ($p<0.05$). Similarly, diet increased other *Lactobacillus spp.* ($F(3, 28) = 5.436$; $p = 0.0045$; Figure 4G), and post hocs revealed that GOS, PDX ($p = 0.0003$) and GOS, PDX, LAC ($p = 0.0199$) diets only significantly increased other *Lactobacillus* spp., and a slight trend was observed in the LAC diet increasing other *Lactobacillus* spp. ($p = 0.12$).

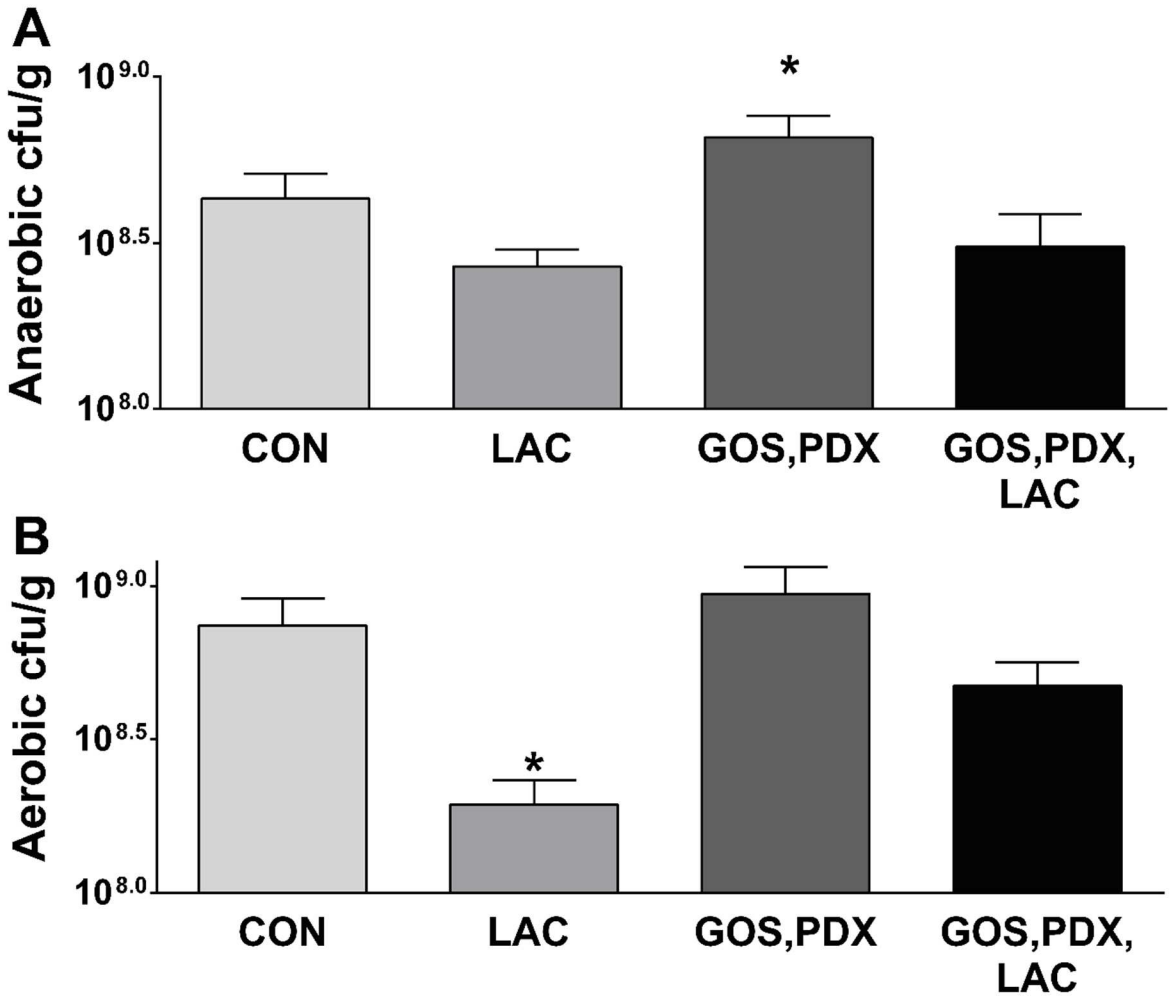
Figure 4



Diet increased *L. rhamnosus* and *L. casei, L. paracasei, L. plantarum, L. fermentum, L. reuteri, L. Sakei.* A) M-RTLV agar. *L. rhamnosus* colonies appear pink or as irregular red colonies surrounded by a white halo (arrows), in contrast to the punctated, deep red of *L. casei, L. paracasei, L. plantarum, L. fermentum, L, reuteri,* and *L. sakei.* B- E) Representative images of the plates by diet. F) *L. rhamnosus* spp. colony forming units (cfu). G) *L. casei, L. paracasei, L. plantarum, L. fermentum, L, reuteri*, and *L. sakei* cfus. Data are represented as mean ± SEM of cfu/ fecal matter (g). * $p < 0.05$. N: 7-9/grp.

Total aerobic and total anaerobic bacteria were also cultured (Supplemental Figure 1). Diet impacted anaerobic (ANOVA ($F(3, 30) = 4.873$; $p = 0.0071$; Figure S1A) and aerobic ($F(3,30) = 4.990$; $p<0.0063$; Figure S1B) bacteria. Post-hocs revealed that LAC diet decreased total aerobic bacteria compared to control diet ($p = 0.0121$), while GOS, PDX diet increased total anaerobic bacteria relative to control diet ($p = 0.0485$).

Supplemental Figure 1



Diet modulated anaerobic and aerobic bacteria. A) total anaerobic and B) aerobic bacteria. Data are represented as mean ± SEM of cfu/ fecal matter (g). * $p < 0.05$. N: 7-9/grp.

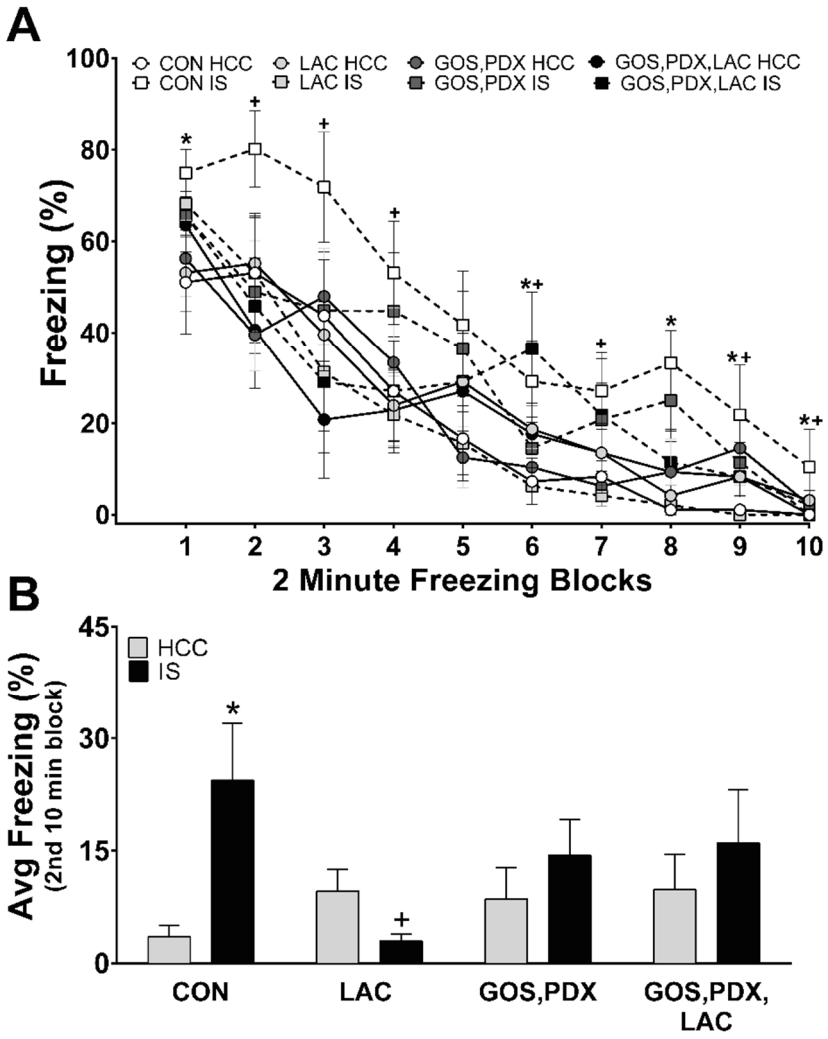
Although diets did not change the impact of stress on corticosterone, blood glucose, or spleen weight, it was possible that changes in bacteria could impact the degree to which stress impacted these measures. Simple regression revealed that increased levels of anaerobic bacteria predicted higher blood glucose following IS ($R = 0.706$, $F(1,13) = 12.884$; $p = 0.0033$), while a similar trend was observed in aerobic bacteria ($R = 0.479$, $F(1,13) = 3.860$; $p = 0.0712$).

d. Diets attenuated stress-induced exaggerated freezing and shuttle box escape deficits

Figure 5A depicts freezing levels for each group across the 20-minute testing session. Repeated measures ANOVA demonstrated that stress increased freezing ($F(1,56) = 3.868$; $p = 0.0542$). Freezing levels decreased across time ($F(9,504) = 67.899$; $p < 0.0001$) in all groups. ANOVA also detected a time x diet interaction ($F(27,504) = 1.743$; $p = 0.0124$), in that IS increased levels of freezing in the Control diet only. Furthermore, LAC, GOS, PDX and GOS, PDX, LAC IS groups froze significantly less than CON diet IS rats at certain time points (see graph for detailed post hoc comparisons).

Figure 5B depicts average freezing for the second 10-minute block of the testing session. Here, a diet x stress interaction was observed in the second 10 minutes of testing ($F(3,56) = 2.873$; $p = 0.0442$), and post hoc comparisons demonstrated that IS increased levels of freezing within the Control diet only during the second half of testing ($p = 0.0031$). In addition, LAC diet IS displayed freezing levels that were significantly lower than CON diet IS rats ($p=0.002$) as well as GOS, PDX, LAC IS rats ($p=0.049$).

Figure 5

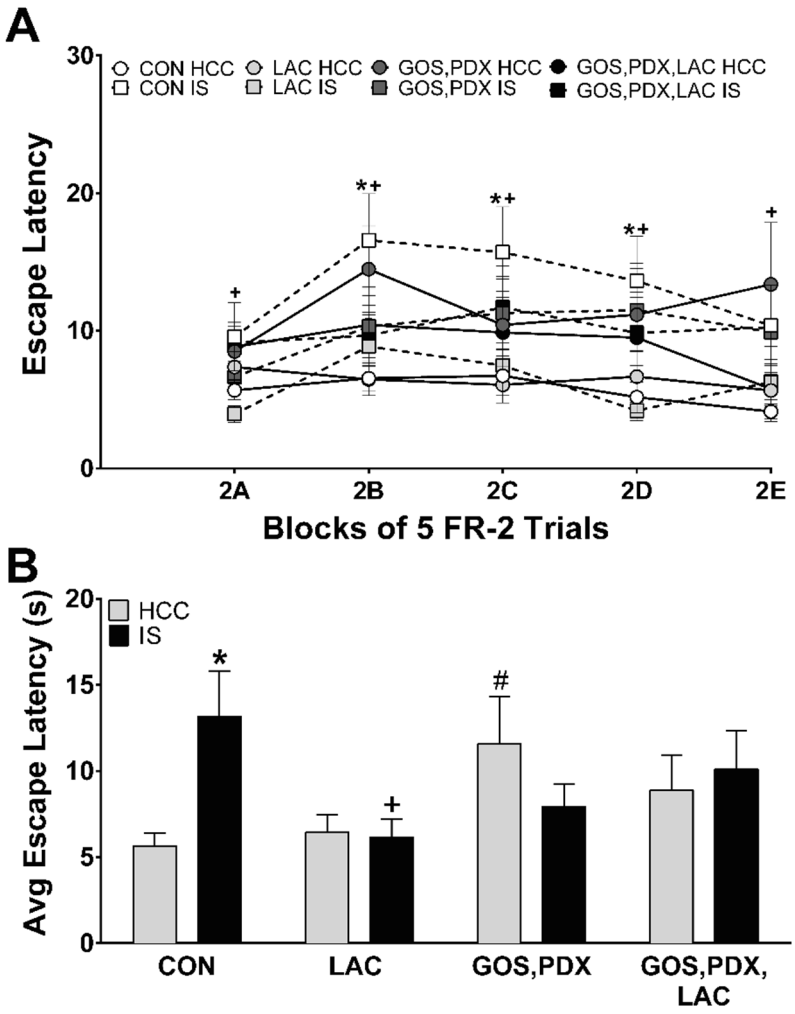


Diets attenuated stress-induced exaggerated freezing. A) Shock-elicited freezing across the 20 min testing session, in 2-minute blocks. B) Average percent time spent freezing, during the second ten minutes of the testing session. Data are represented as mean ± SEM. */+ $p < 0.05$. * indicates a difference between HCC and IS, + indicates difference between C IS and experimental diet IS. N: 8/grp.

Figure 6A depicts escape latencies for each group across testing session; each block represents an average of 5 FR-2 trials. Repeated measures ANOVA demonstrated that escape latency initially rose then fell over the course of testing ($F(4, 212) = 4.717$; $p < 0.011$). Additionally, a diet x stress interaction was detected ($F(3,53) = 2.951$; $p=0.0409$)), in that IS increased escape latencies in the CON diet only ($p=0.0198$; see graph for detailed post hoc comparisons).

Figure 6B depicts average escape latency; similarly, IS increased average escape latency in the CON diet only. In addition, only LAC diet IS rats displayed average escape latencies that were significantly lower than CON diet average escape latencies. Finally, GOS, PDX diet home cage controls displayed escape latencies that were significantly elevated compared with CON diet home cage controls.

Figure 6.



Diets attenuated stress-induced shuttle box escape deficits. A) escape latencies across time, in 5 blocks of 5 trials. B) average escape latency across the entire testing session. Data are represented as mean ± SEM. */+/# $p < 0.05$. * indicates a difference between HCC and IS, + indicates a difference between C IS and experimental diet IS, # indicates a difference between C HCC and experimental diet HCC. N: 6-8/grp.

e. Diets attenuated stress-evoked increases in cfos mRNA within the DRN

Figure 7A depicts *cfos* mRNA expression in rostral, mid, and caudal regions of the DRN. *cfos* mRNA levels differed significantly across rostral, mid and caudal subregion ($F(2,150) = 43.517$; $p < 0.0001$). IS increased *cfos* mRNA in the rostral, mid and caudal subregions of the DRN ($F(1,150) = 926.782$; $p < 0.0001$). Additionally, an effect of diet ($F(3,150) = 6.802$; $p<0.0002$), and a diet x stress interaction ($F(3,150) = 8.172$; $p <0.0001$) revealed that the diets differentially attenuated the impact of stress on *cfos* expression within each region. Within the rostral DRN, the stress-evoked increase in *cfos* mRNA was attenuated in the GOS, PDX group. Within the mid DRN, all three experimental diets attenuated stress-evoked increases in *cfos* mRNA, compared to CON diet IS rats. Within the caudal DRN, no significant attenuation of the stress effects was observed.

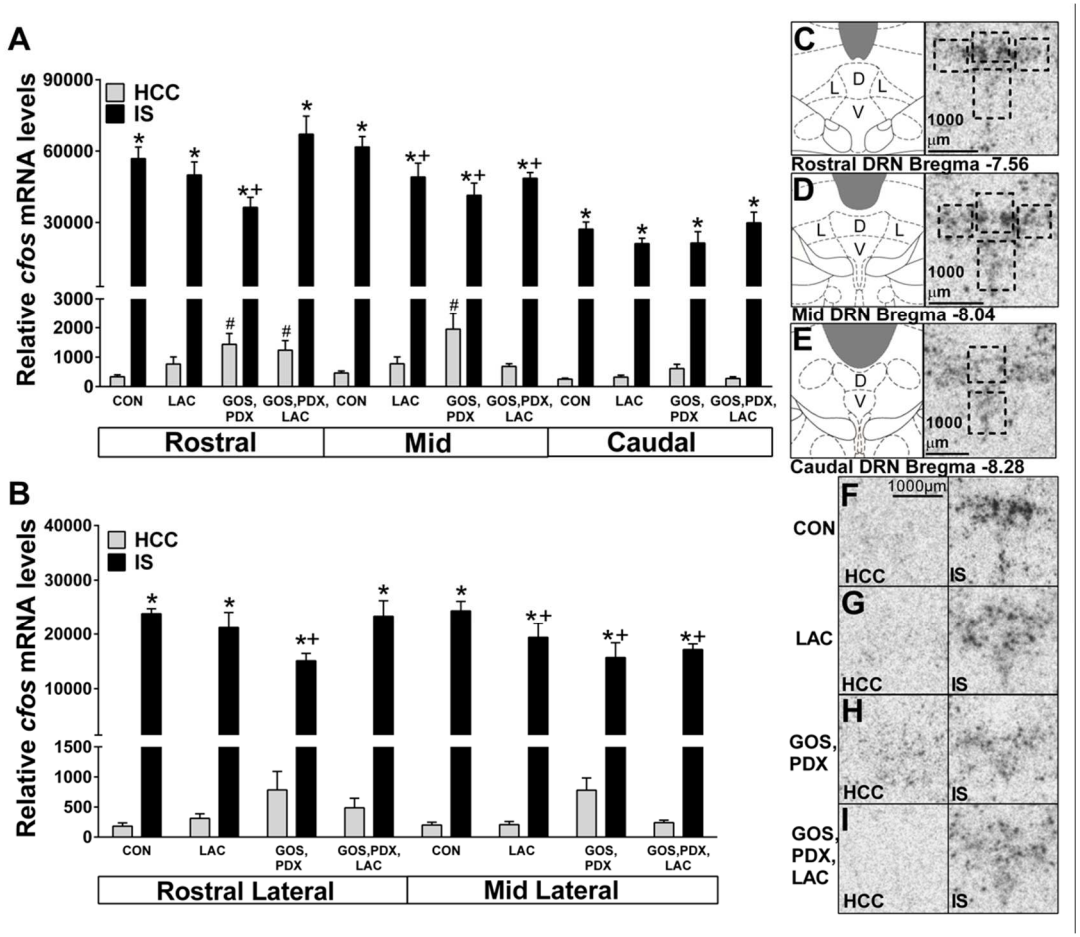
To examine the possible impact of diet on *cfos* expression in HCC groups only, ANOVA revealed that *cfos* mRNA differed by subregion ($F(2,75) = 7.592$, and that diet impacted basal *cfos* mRNA levels within the DRN ($F(3,75) = 8.628$; $p<0.0001$), in that GOS, PDX diet increased basal *cfos* levels within the DRN overall (Fisher's PLSD; $p<0.0001$). Specifically, post hocs within subregions revealed that *cfos* mRNA levels were significantly increased by GOS, PDX ($p = 0.003$) and GOS, PDX, LAC ($p = 0.016$) diets only. In the mid DRN, post hoc revealed that GOS, PDX diet increased *cfos* mRNA compared to control diet ($p<0.0001$).

Figure 7B depicts *cfos* mRNA expression in the rostral and mid lateral wings of the DRN. Stress increased *cfos* mRNA overall in the rostral and mid lateral wings ($F(1,100) = 677.863$; $p < 0.0001$). Additionally, a main effect of diet ($F(3,100) = 5.037$; $p = 0.0027$), and a diet x stress interaction ($F(3,100) = 6.636$; $p = 0.0004$) revealed that within the rostral lateral DRN, the

increase in *cfos* mRNA following stress was attenuated in the GOS, PDX group only, compared to CON diet IS rats. Within the mid lateral wings, all three experimental diets attenuated stress-evoked increases in *cfos* mRNA.

ANOVA in the HCC groups only revealed a main effect of diet in the lateral wings ($F(3,51) = 5.987$; $p = 0.0014$), in that just the GOS, PDX diet increased basal levels of *cfos* mRNA compared to control diet.

Figure 7.



Diets attenuated stress-evoked increases in *cfos* mRNA within the DRN. A) *cfos* mRNA expression in rostral, mid, and caudal regions of the DRN, summed across dorsal and ventral regions, in arbitrary units. B) *cfos* mRNA expression in the rostral and mid lateral wings of the DRN. C-E) Atlas images and corresponding autoradiographs show *in situ* hybridization of *cfos* in the rostral, mid and caudal DRN, respectively. Boxes represent regions that were sampled for quantification. F-I) Representative images of the mid DRN for each diet, HCC and IS groups. Data are represented as mean ± SEM. */+/# $p < 0.05$. * indicates a difference between HCC and IS, + indicates a difference between C IS and experimental diet IS, # indicates a difference between C HCC and experimental diet HCC. N: 6-8/grp.

f. Impact of stress and diet on cfos mRNA within central stress circuits

NTS and LC. IS increased *cfos* mRNA, regardless of diet, within the NTS ($F(1,25) = 31.514$; $p<0.0001$; Figure 8M) and the LC ($F(1,34) = 69.485$; $p<0.0001$; Figure 8N). Diet had no impact on these regions.

Habenula and BNST. Stress increased *cfos* mRNA in all diets within the habenula ($F(1,50) = 105.451$; $p<0.0001$; Figure 8O) and diet had no impact.

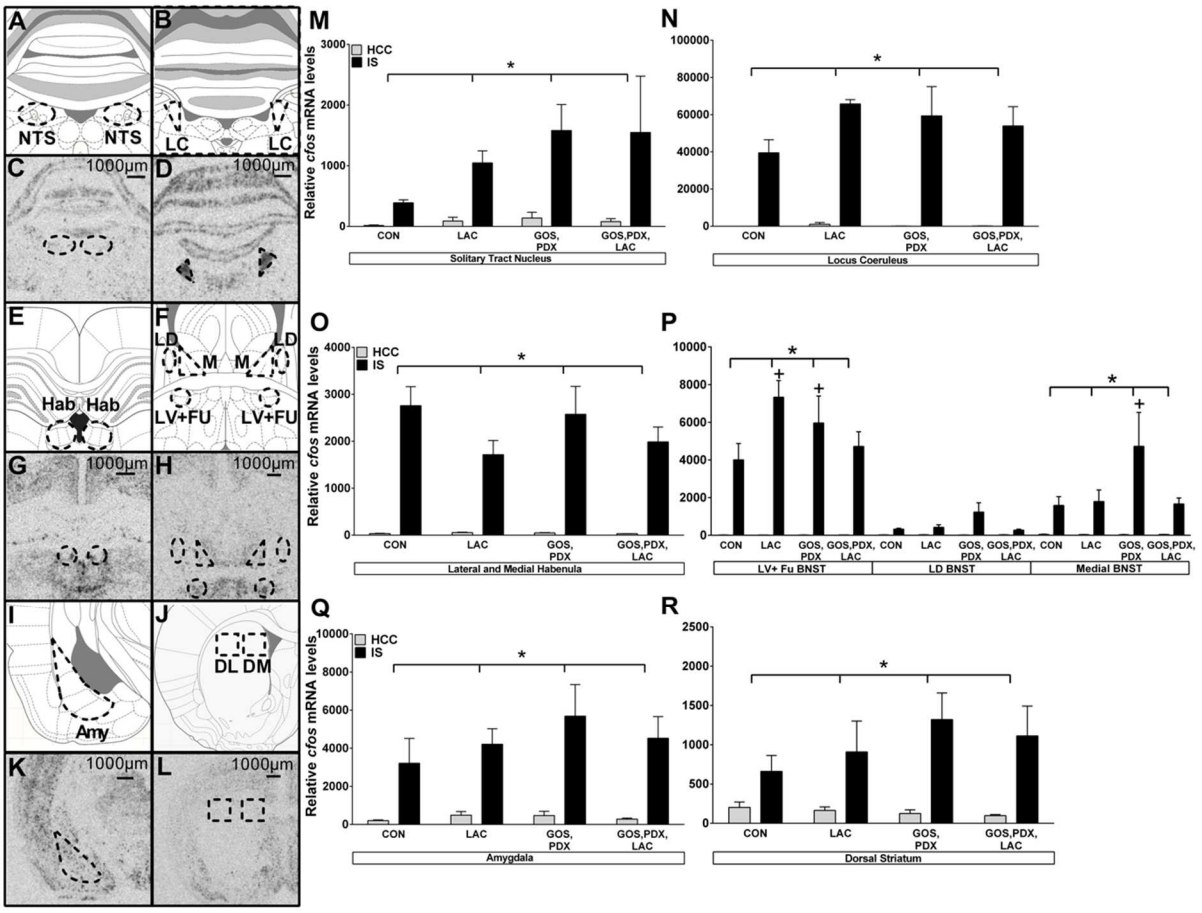
In the BNST, ANOVA revealed that *cfos* mRNA levels differed between subregions ($F(2,137) = 37.750$; $p<0.0001$; Figure 8P). ANOVA also revealed a main effect of diet ($F(3,137) = 3.986$; $p=0.0093$, stress ($F(1, 137) = 147.909$; $p<0.0001$), and a diet by stress interaction ($F(2,137) = 4.04$; $p = 0.0087$). Fisher's PLSD revealed that GOS, PDX ($p=0.0289$) and LAC ($p=0.0291$) diets increased *cfos* mRNA overall, and post hocs within subregions further revealed that IS increased *cfos* mRNA expression within all diets in the lateral ventral and fusiform nuclei ($p<0.0001$). Additionally, in the lateral ventral and fusiform nuclei, stress potentiated *cfos* mRNA in LAC ($p<0.0001$) and GOS, PDX ($p = 0.017$) IS groups, in comparison to the CON diet IS group. Similarly, stress potentiated *cfos* expression in the GOS, PDX IS ($p = 0.0002$) group compared to CON diet IS in the medial nuclei.

A similar pattern was observed in the medial BNST; stress increased *cfos* expression in all diets (CON, $p = 0.05$; LAC, $p = 0.0316$; GOS, PDX, $p = 0.0001$; GOS, PDX, LAC, $p = 0.033$). Post hocs further revealed that stress potentiated *cfos* mRNA expression in the GOS, PDX diet only, in that stress significantly increased *cfos* mRNA only in the GOS, PDX IS rats compared to CON diet IS rat ($p = 0.0002$).

Striatum and Amygdala. Stress increased *cfos* mRNA in all diets within the amygdala ($F(1,50) = 40.639$; $P<0.0001$; Figure 8Q) as well as the striatum ($F(1,50) = 24.476$; $p<0.0001$; Figure 8R), and diet had no impact.

ANOVA on HCC groups only revealed no significant differences in basal *cfos* expression between diets.

Figure 8



Impact of stress and diet on *cfos* mRNA within central stress circuits. Atlas images and corresponding autoradiographs showing *in situ* hybridization of *cfos* in the NTS (A, C), LC (B, D), lateral and medial habenula (E, G), BNST (by subregion: lateral ventral and fusiform; LV + Fu, lateral dorsal; LD, and medial; F, H), amygdala (I, K) and dorsal striatum (J, L). Areas outlined on the autoradiographs indicate regions sampled for quantification. *cfos* mRNA in all diets, following IS in the NTS (M), LC (N), habenula (O), BNST (P), amygdala (Q), and dorsal striatum, summed across dorsal lateral and dorsal medial regions. Data are represented as mean ± SEM. */+ $p < 0.05$. * indicates a main effect of stress, + indicates a difference between C IS and experimental diet IS. N/grp: NTS: 3-6/grp; LC: 3-8/grp; habenula: 6-8/grp; LV+FU BNST: 5-8/grp; LD BNST: 6-8/grp; medial BNST: 6-8/grp amygdala: 6-7/grp; dorsal striatum: 7-8/grp.

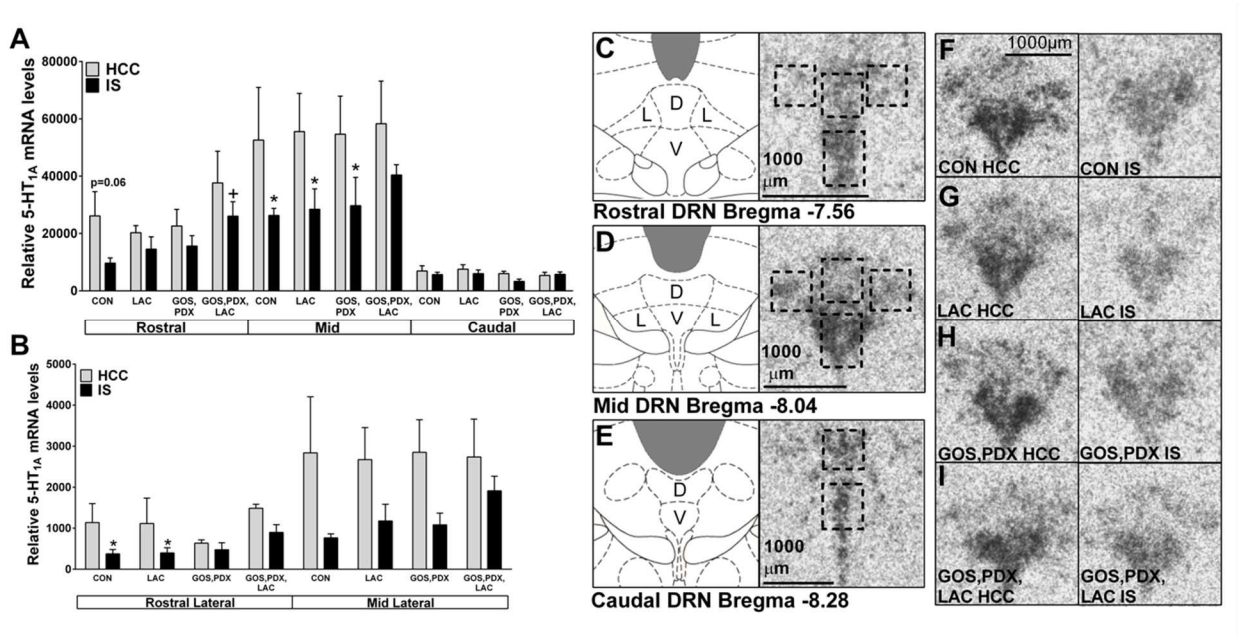
g. Diets attenuated stress-evoked decreases in 5-HT1AR mRNA expression within the DRN

Figure 9A depicts 5-HT1AR mRNA expression in the rostral, mid, and caudal DRN. 5-HT1AR mRNA expression differed significantly by subregion ($F(2,130) = 34.971$; $p < 0.0001$). Stress decreased 5-HT1AR mRNA expression overall (Stress: $F(1,130) = 10.614$; $p = 0.0014$), and a region by stress interaction was observed ($F(2,130) = 3.278$; $p = 0.0409$), revealing that the diets differentially attenuated the impact of stress on 5-HT1AR mRNA expression within each region. Within the rostral DRN, a trend ($p = 0.06$) toward a stress-evoked decrease within the control diet was observed, but not in the experimental diets. Additionally, in the mid DRN, stress decreased 5-HT1AR mRNA expression in all diets except the GOS, PDX, LAC diet. No stress or diet effects were observed within the caudal DRN.

Figure 9B depicts 5-HT1AR mRNA expression in the rostral and mid lateral wings of the DRN. 5-HT1AR mRNA expression differed significantly by subregion ($F(1,80) = 11.447$; $p = 0.0011$). Stress ($F(1,80) = 8.916$; $p = 0.0037$) decreased 5-HT1AR mRNA expression in the rostral lateral wings, and post hocs revealed that stress significantly decreased 5-HT1AR mRNA expression within the control and LAC diets only.

ANOVA on HCC groups only revealed no significant differences in basal 5-HT1AR mRNA expression between diets.

Figure 9



Diets attenuated stress-evoked decreases in 5-HT1AR mRNA expression within the DRN. A) 5-HT1AR mRNA expression in the rostral, mid, and caudal DRN, summed across dorsal and ventral regions. B) 5-HT1AR mRNA expression in the rostral and mid lateral wings of the DRN. C-E) Atlas images and corresponding autoradiographs showing *in situ* hybridization of 5-HT1AR in the rostral, mid, and caudal DRN. Areas outlined on the autoradiographs indicate regions sampled for quantification. F-I) Representative images of the mid DRN for each diet, non-stress and stress groups. Data are represented as mean ± SEM: * $p < 0.05$. * indicates a difference between HCC and IS, + indicates a difference between C IS and experimental diet IS. N/grp: rostral: 4-8/grp; mid: 6-8/grp; caudal: 3-7/grp; rostral lateral: 3-7/grp; mid lateral: 6-8/grp.

h. Stress and diet modulated 5-HT2CR in DRN projection regions

Supplemental table 1 depicts 5-HT2CR mRNA expression with various subregions of the dorsal striatum and the amygdala as mean ± SEM., and supplemental figure 2 indicates regions sampled for quantification.

5-HT2CR mRNA differed by subregion within the amygdala ($F(2,150) = 767.457$; $p<0.0001$). ANOVA also revealed an effect of diet ($F(3,150) = 3.111$; $p = 0.0282$), and a diet by stress interaction ($F(3,150) = 2.909$; $p = 0.0366$). Fisher's PLSD revealed that across stress groups and subregions, 5-HT2CR mRNA was higher in the GOS, PDX, LAC relative to CON diet overall ($p = 0.023$). Furthermore, region-specific post hocs revealed that within the dorsolateral amygdala, IS decreased 5-HT2CR mRNA in CON diet rats only ($p = 0.01$).

ANOVA on HCC only revealed basal differences in 5-HT2CR mRNA within the amygdala, in that 5-HT2CR mRNA levels differed across subregions ($F(2,78) = 391.382$; $p<0.0001$) and diet ($F(3,78) = 3.404$; $p = 0.0217$). Fisher's PLSD revealed that 5-HT2CR mRNA in LAC HCC rats was decreased compared to CON HCC ($p = 0.01$), and subregion specific post hocs revealed this was specific to the dorsolateral amygdala ($p = 0.009$).

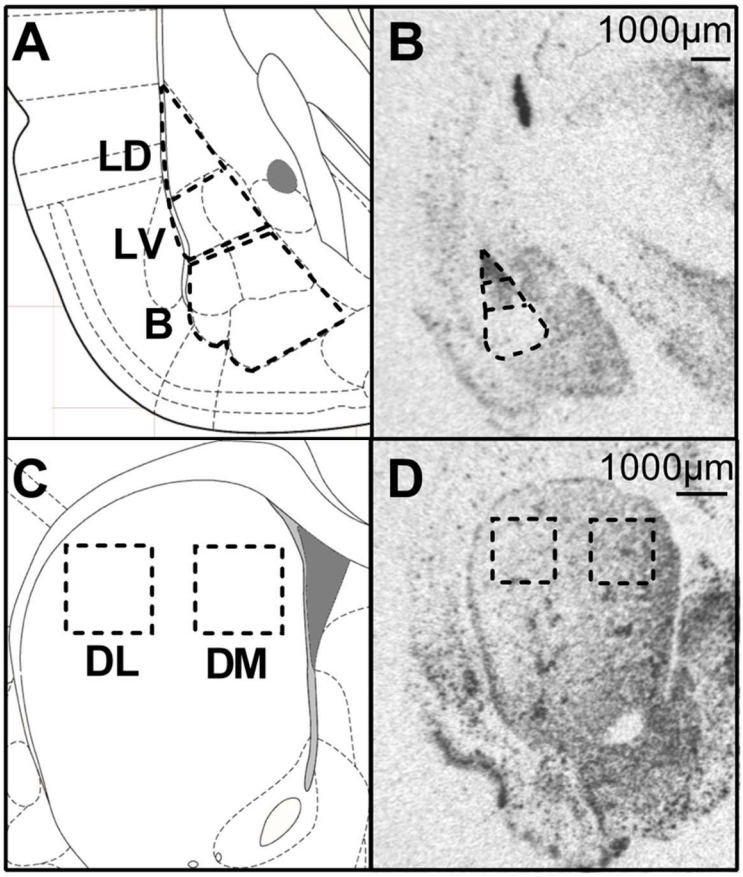
No significant effects of diet or stress were observed within the dorsal striatum.

Supplemental table 1

Region	Con HCC	Con IS	LAC HCC	LAC IS	GOS,PDX HCC	GOS,PDX IS	GOS,PDX,LAC HCC	GOS,PDX,LAC IS
Amygdala-LD	21218.88 ± 1060.89	18086.75 ± 1204.92 *	17492.11 ± 1864.09 #	19735.12 ± 1631.32	21877.72 ± 998.57	19312.65 ± 796.97	20861.91 ± 1626.69	19656.93 ± 1063.45
Amygdala-LV	12167.17 ± 808.59	9991.91 ± 1345.12	10123.63 ± 891.91	11548.45 ± 704.44	12043.58 ± 806.927	10872.43 ± 1195.23	13843.705 ± 1363.72	14263.76 ± 555.78
Amygdala-Basal	1060.41 ± 145.90	834.69 ± 79.32	979.77 ± 96.28	968.13 ± 132.21	777.64 ± 112.15	879.19 ± 122.12	1017.16 ± 171.73	1077.85 ± 179.51
Striatum-DL	3213.33 ± 233.89	3065.63 ± 360.18	3455.36 ± 374.65	2951.99 ± 356.24	2926.26 ± 179.06	3217.33 ± 252.78	3498.44 ± 337.29	2907.68 ± 428.82
Striatum-DM	36060.61 ± 1670.13	36119.51 ± 977.18	35077.64 ± 2058.78	28505.44 ± 2201.02	33735.40 ± 2274.19	32043.27 ± 2658.77	38105.41 ± 2937.34	33780.59 ± 3962.02

Stress and diet modulated 5-HT2CR in DRN projection regions. 5-HT2CR mRNA expression in the lateral dorsal (LD), lateral ventral (LV) and basal amygdala and dorsal lateral (DL) and dorsal medial (DM) striatum. Data are represented as mean ± SEM: */# $p < 0.05$. * indicates a difference between HC and IS, # indicates a difference between C HCC and experimental diet HCC. N/grp throughout amygdala: 6-8/grp; throughout striatum: 7-8/grp.

Supplemental figure 2



Atlas images and corresponding audio radiographs showing *in situ* hybridization of 5-HT2CR in the amygdala (A) and dorsal striatum (B). Areas outlined in the autoradiographs indicate regions sampled for quantification.

i. Stress but not diet modulated $GABA_{A\alpha2}R$ subunits within central stress circuits

Previous work demonstrated that probiotic *Lactobacillus* spp. were capable of altering gene expression for GABA receptor subunits (Bravo et al., 2011). Supplemental table 2 depicts $GABA_{A\alpha2}R$ mRNA expression with various subregions of the hippocampus and amygdala as mean ± SEM. No significant effects of diet or stress were observed within the amygdala. Within the hippocampus, mRNA differed by subregion ($F(3,204) = 934.540$; $p<0.0001$). ANOVA further revealed an effect of stress ($F(1,204) = 5.692$; $p = 0.018$), with a region by stress interaction ($F(3,204)$; $p = 0.0492$). Fisher's PLSD showed that within the dentate, stress decreased $GABA_{A\alpha2}R$ mRNA expression ($p = 0.015$).

ANOVA on HCC groups only revealed no significant differences in basal $GABA_{A\alpha2}R$ mRNA expression between diets.

Supplemental table 2

Region	Con HCC	Con IS	LAC HCC	LAC IS	GOS,PDX HCC	GOS,PDX IS	GOS,PDX,LAC HCC	GOS,PDX,LAC IS
Hipp-DG	23744.21 ± 1555.97	21373.97 ± 1516.63 *	24319.64 ± 1229.31	21682.67 ± 1325.53	24838.89 ± 1269.84	23497.34 ± 1821.65	25970.36 ± 2800.43	23379.68 ± 2048.81
Hipp-CA3	13800.19 ± 831.04	12446.50 ± 1360.74	15032.94 ± 1242.97	13332.17 ± 944.21	13192.80 ± 722.27	13772.81 ± 1240.75	13664.65 ± 1295.06	13661.10 ± 998.20
Hipp-CA2	1814.96 ± 100.16	1561.85 ± 159.67	2098.00 ± 304.94	1879.04 ± 154.16	1804.48 ± 107.38	1733.07 ± 165.29	1860.875 ± 168.49	1946.33 ± 173.64
Hipp-CA1	1206.99 ± 112.48	1109.15 ± 204.63	1129.67 ± 96.43	1020.30 ± 120.37	1142.65 ± 45.27	1142.20 ± 135.24	1295.87 ± 188.65	1130.82 ± 90.61
Amygdala	77138.29 ± 4796.11	58771.80 ± 9958.63	60875.93 ± 9706.18	72442.06 ± 9556.86	69410.35 ± 8719.43	59588.27 ± 2962.89	68640.408 ± 7696.22	77057.20 ± 8461.17

Stress but not diet modulated $GABA_{A\alpha2}R$ subunits within central stress circuits. $GABA_{A\alpha2}$ mRNA expression in the DG, CA3, CA2 and CA1 of the hippocampus and amygdala. Data are represented as mean ± SEM: * $p < 0.05$ indicates a difference between HCC and IS. N: 6-8/grp.

j. Stress and diet modulated BDNF mRNA expression within central stress circuits

Figure 10 depicts BDNF mRNA expression with various subregions of the PFC and hippocampus. Within the PFC, mRNA differed across subregions ($F(2,153) = 10.922$; $p<0.0001$; Figure 10A), and ANOVA also revealed an effect of stress ($F(1,153) = 130.754$; $p<0.0001$), in that IS increased BDNF mRNA expression in all diets and across all subregions (In the CG, CON HCC v. IS $p=0.0037$, LAC HCC v. IS $p=0.0077$, GOS, PDX HCC v. IS $p = 0.02$, GOS, PDX, LAC HCC v. IS $p = 0.0064$; In the IL, CON HCC v. IS $p=0.0004$, LAC HCC v. IS $p = 0.0059$, GOS, PDX HCC v. IS $p = 0.0016$, GOS, PDX, LAC HCC v. IS $p = 0.0042$; In the PL, CON HCC v. IS $p<0.0001$, LAC HCC v. IS $p = 0.0001$, GOS, PDX HCC v. IS $p = 0.0002$, GOS, PDX, LAC HCC v. IS $p<0.0001$). In addition, Fisher's PLSD revealed that across subregions and stress groups, GOS, PDX ($p = 0.05$) and GOS, PDX, LAC diet (0.009) increased BDNF mRNA expression relative to CON diet. Region specific post hocs revealed that only GOS, PDX, LAC diet increased BDNF gene expression within the CG ($p = 0.05$) and PL ($p = 0.0092$), specifically.

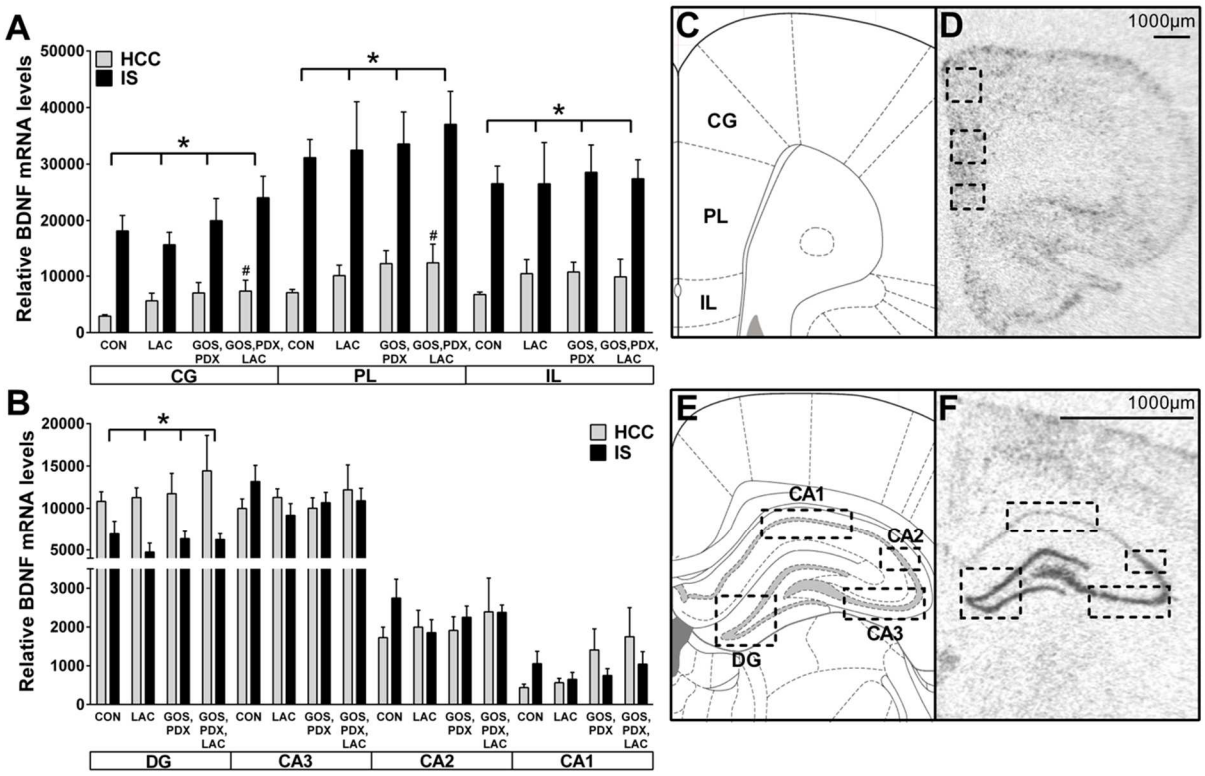
ANOVA on HCC only revealed basal differences in BDNF mRNA expression. BDNF mRNA differed by region ($F(2,78) = 4.503$; $p = 0.0141$), and by diet ($F(3,78) = 4.102$; $p = 0.0093$). Fisher's PLSD showed that GOS, PDX, LAC diet increased BDNF mRNA expression relative to CON diet overall ($p = 0.0009$), and region specific post hocs revealed that BDNF mRNA increased in the GOS, PDX, LAC HCC group relative to CON HCC ($p = 0.009$) in the PL.

Within the hippocampus, BDNF mRNA expression differed by subregion ($F(3,216) = 88.113$; $p<0.0001$; Figure 10B), and by stress ($F(1,216) = 10.546$; $p<0.0014$). ANOVA also revealed a region x stress interaction ($F(3,216) = 5.921$; $p<0.0007$). Subregion specific post hocs

revealed that within the dentate, stress decreased BDNF mRNA expression in all diets (CON HCC v. IS $p = 0.009$, LAC HCC v. IS $p = 0.0271$, GOS, PDX HCC v. IS $p = 0.02$, GOS, PDX, LAC HCC v. IS $p = 0.0013$); no significant effects of diet or stress were observed within the CA3, CA2, and CA1.

ANOVA on HCC groups only revealed no significant differences in basal BDNF mRNA expression between diets within the hippocampus.

Figure 10

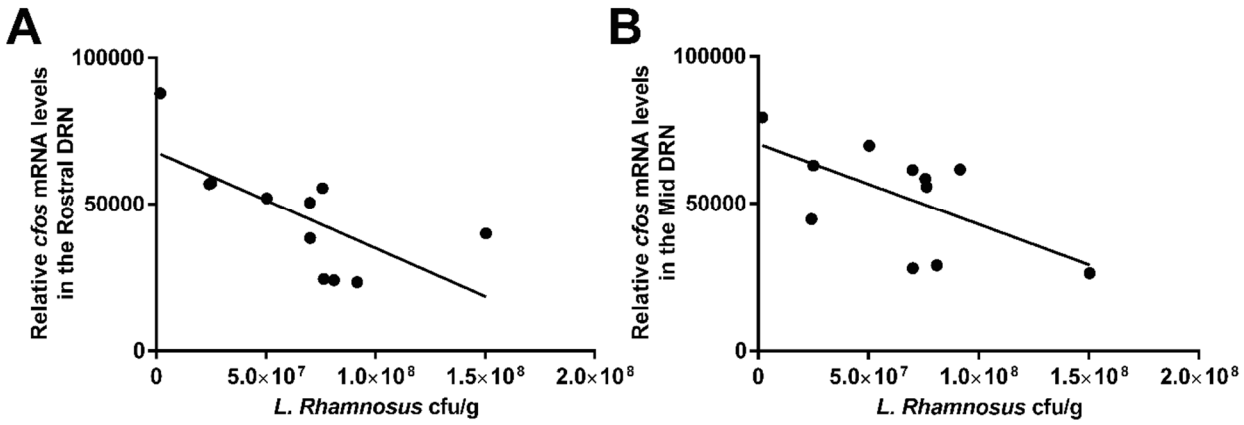


Stress and diet modulated BDNF mRNA expression within central stress circuits. A) BDNF mRNA expression in the CG, PL and IL of the PFC. B) BDNF mRNA expression in the DG and CA3, CA2 and CA1 fields of the hippocampus. Atlas images and corresponding autoradiographs show *in situ* hybridization for BDNF in the CG, PL and IL of the PFC (C, D) and the DG and CA3, CA2 and CA1 fields of the hippocampus (E, F). Areas outlined in the autoradiographs indicate regions sampled for quantification. Data are represented as mean ± SEM: */# $p < 0.05$. * indicates a main effect of stress, # indicates a difference between C HCC and experimental diet HCC. N/grp: PFC: 6-8/grp; DG: 5-7/grp; CA3/CA2: 7-8/grp; CA1: 6-8/grp.

k. Lactobacillus spp. predict stress-protective alterations in mRNA expression within the DRN

Lactobacillus spp. predicted the degree to which diets attenuated stress-evoked increases in *cfos* mRNA within the DRN. Increased levels of *L. Rhamnosus* predicted decreased levels of *cfos* mRNA within IS groups within the rostral (simple regression; $R = 0.683$, $F(1,9) = 7.879$; $p = 0.0205$; Figure 11A), and a trend was observed in the rostral lateral wings ($R = 0.588$, $F(1,8) = 4.235$; $p = 0.07$) of the DRN. Similarly, increased levels of *L. Rhamnosus* predicted decreased *cfos* mRNA within IS groups within the mid DRN ($R = 0.610$, $F(1,9) = 5.337$; $p = 0.0462$; Figure 11B), while a trend was observed with other *Lactobacillus* spp. ($R = 0.584$, $F(1,9) = 4.64$; $p=0.06$).

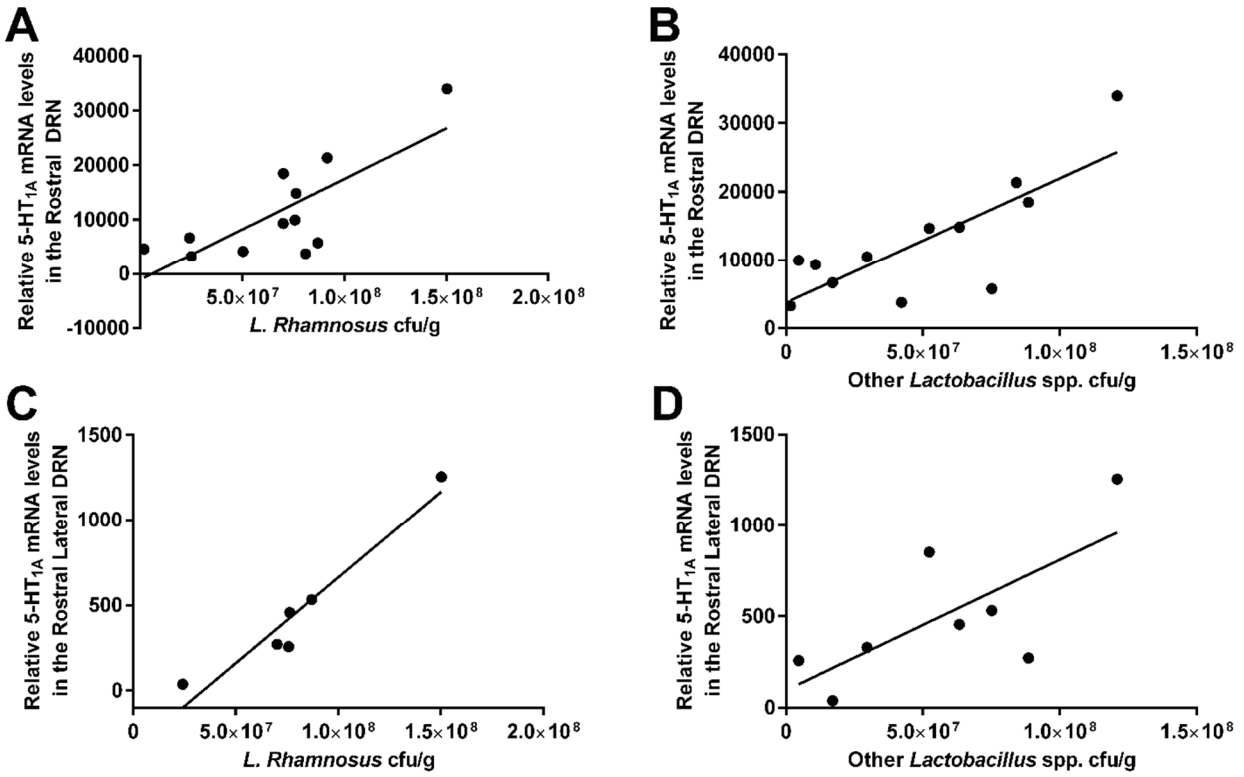
Figure 11



Lactobacillus spp. predicted stress-protective alterations in *cfos* mRNA expression within the DRN. Simple regressions within IS groups of each diet, for A) *L. Rhamnosus* (cfu/g) and *cfos* mRNA expression in the rostral DRN and B) *L. Rhamnosus* and *cfos* mRNA expression in the mid DRN. N: 11/grp.

Increased levels of *Lactobacillus* spp. predicted the degree to which diets attenuated stress-evoked decreases in 5-HT1AR mRNA expression within the DRN. Higher levels of *L. Rhamnosus* (R=0.770, F (1,10) = 14.548; p = 0.0034; Figure 12A) as well as other *Lactobacillus* spp. (R = 0.791, F (1,10) = 16.733; p = 0.0022; Figure 12B) predicted increased levels of 5-HT1AR mRNA within IS groups within the rostral as well as the rostral lateral wings (R = 0.965, F (1,4) = 53.943; p = 0.0018, Figure 12C; R=0.717, F (1,6) = 6.355; p = 0.0452, Figure 12D, respectively).

Figure 12



Lactobacillus spp. predict stress-protective alterations in 5-HT1AR mRNA expression within the DRN. Simple regressions within IS groups of each diet, for A) *L. Rhamnosus* and 5-HT1AR mRNA expression in the rostral DRN, B) *Lactobacillus* spp. and 5-HT1AR mRNA expression in the rostral DRN, C) *L. Rhamnosus* and 5-HT1AR mRNA expression in the rostral lateral wings, D) *Lactobacillus* spp. and 5-HT1AR mRNA expression in the rostral lateral wings. N/grp: A) & B) n: 12/grp; C) n: 6/grp; D) n: 8/grp.

l. Bacteria and markers of sympathetic and HPA activation predict stress-induced alterations in gene expression

To determine whether levels of bacteria impacted the degree to which IS altered gene expression, simple regressions were run between bacterial culture data and gene expression within IS groups. Results are summarized in Supplemental Table 3. Briefly, increased levels of aerobic bacteria predicted greater increases in *cfos* mRNA expression following IS within the habenula, LD and medial BNST, and dorsal striatum; increased levels of anaerobic bacteria predicted greater increases in *cfos* mRNA expression following IS within the LD BNST and dorsal striatum; increased levels of *L. rhamnosus* predicted greater increases in BDNF mRNA expression following IS within the CG of the PFC.

Supplemental Table 3

Bacteria	Brain Region & mRNA Probe	R	P Value
Aerobic	*cfos* in Habenula	0.610	0.0158
	cfos in LD BNST	0.839	0.0012
	cfos in Medial BNST	0.633	0.0203
	cfos in Dorsal Striatum	0.652	0.0216
Anaerobic	*cfos* in LD BNST	0.734	0.0101
	cfos in Dorsal Striatum	0.640	0.0249
L. *rhamnosus*	BDNF in CG PFC	0.673	0.0468

Bacteria predict stress-induced alterations in gene expression. Simple regressions within IS groups of each diet, for aerobic, anaerobic and *L. Rhamnosus* with *cfos* and BDNF mRNA expression in various brain regions. N: 9-15/grp.

To examine the relationship between IS-induced alterations in corticosterone, blood glucose, or spleen weight and altered gene expression in the brain, simple regressions were run between these markers of sympathetic and HPA activity and gene expression within IS groups. Results are summarized in Supplemental Table 4. Briefly, higher blood glucose predicted greater increases in *cfos* mRNA expression following IS within the habenula, LD and medial BNST, and dorsal striatum and 5-HT1AR mRNA within the mid DRN; lower spleen weight predicted greater increases in *cfos* mRNA expression following IS within NTS and LC; higher corticosterone predicted greater increases in $GABA_{A\alpha 2}R$ mRNA expression within the CA1 of the hippocampus.

Supplemental Table 4.

Measures of Sympathetic & HPA Activity	Brain Region & mRNA Probe	R	P Value
Blood Glucose	*cfos* in Habenula	0.524	0.0030
	cfos in LD BNST	0.454	0.0197
	cfos in Medial BNST	0.537	0.0032
	cfos in Dorsal Striatum	0.455	0.0170
	$5HT_{1A}$ in Mid DRN	0.387	0.0460
Spleen Weight	*cfos* in NTS	0.765	0.0014
	cfos in LC	0.471	0.0232
Corticosterone	$GABA_{Aa2}$ in CA1 of Hippocampus	0.389	0.0370

Markers of sympathetic and HPA activation predict stress-induced alterations in gene expression. Simple regressions within IS groups of each diet, for blood glucose, spleen weight and corticosterone (CORT) with *cfos,* 5-HT1AR and $GABA_{A\alpha2}$ mRNA expression in various brain regions. N/grp: blood glucose/ habenula: 30/grp; blood glucose/ lateral dorsal (LD) BNST: 26/grp; blood glucose/ medial BNST: 28/grp; blood glucose/ dorsal striatum (DS): 27/grp; blood glucose/ medial DRN: 27/grp; spleen weight/ NTS: 14/grp; spleen weight/ LC: 23/grp; CORT/ CA1: 29/grp.

v. Discussion

Early life is a uniquely plastic time for brain and microbial development, and may represent a sensitive window during which nutrition and gut bacteria can influence brain and behavior. Our results demonstrate that early life consumption of diets containing the prebiotic blend GOS, PDX and/or LAC distinctly attenuate the expression of stress-induced learned helplessness behaviors and furthermore, uniquely modulate gene expression within circuits important for stress resistance and affective behavior.

There were notable differences in the degrees by which diets protected against learned helplessness. LAC alone produced the greatest degree of protection against IS-induced increases in freezing behavior. In all diets, freezing was slightly elevated in home cage control groups, though no significant differences between home cage controls were detected. Similarly, LAC alone produced the greatest protection against IS-induced increases in escape latency. Home cage controls in diets supplemented with GOS and PDX displayed elevated escape latency, with a significant basal difference between GOS, PDX and CON diet. Tarr et al. (2015) demonstrated patterns suggestive of a similar trend within their open field task; home cage control mice fed prebiotic diets displayed a visible reduction in time spent in the center of the field, compared to non-stressed mice fed the control diet. These patterns of behavior within non-stressed controls are surprising, and more research is needed to elucidate the distinct behavioral effects of different prebiotic diet combinations, basally as well as after stress.

As expected, we demonstrated a drastic increase in *cfos* mRNA expression within the DRN following IS (Grahn et al., 1999; Greenwood et al., 2005a; Greenwood et al., 2003a). This *cfos* increase is consistent with IS-induced hyperactivation of DRN neurons; hyper-activation and subsequent sensitization of DRN 5-HT neurons is necessary (Maier et al., 1995b; Maier et

al., 1994) and sufficient (Maier et al., 1995a) for LH behaviors 24 hours later. Interestingly, LAC alone, GOS, PDX alone, and GOS, PDX, LAC diets all attenuated IS-evoked *cfos* activity in the DRN, in a sub-region specific manner. The most robust effects occurred in the mid DRN; diets similarly attenuated stress-evoked *cfos* mRNA within the mid and mid lateral DRN. GOS, PDX diet alone also attenuated stress-evoked *cfos* mRNA in the rostral and rostral lateral DRN. Exercise and behavioral control produce similar patterns, in that these stress-protective manipulations also attenuate *cfos* in the DRN following IS (Grahn et al., 1999; Greenwood et al., 2005a; Greenwood et al., 2003a). The current data are thus consistent with the idea that prebiotic diets may constrain DRN activity during IS, which is a mechanism known to be able to confer stress resistance (Maier et al., 1995b; Maier et al., 1994).

We did not measure *cfos* mRNA in distinct DRN cell types, however, we expect that the majority of IS-induced *cfos* observed within the boundaries of the mid DRN was expressed by 5-HT neurons, as was found in prior work (Greenwood et al., 2003a). This would also be consistent with anatomical observations demonstrating that the dorso-ventral aspect of the mid DRN contains densely packed 5-HT cells and is largely devoid of other cell types (Day et al., 2004). Regardless of what cell type our DRN *cfos* represents, our data nonetheless demonstrate that diets containing prebiotics and/ or LAC are capable of reducing the impact of stress in the DRN.

Furthermore, GOS, PDX and LAC diet only attenuated the IS-evoked decreases in 5-HT1AR mRNA expression in the DRN. The 5-HT1AR is an important regulator of DRN activity; its activation reduces neuronal firing and 5-HT synthesis (Chen & Penington, 1996; Valdizan et al., 2010), providing negative feedback. IS-induced desensitization of 5-HT1AR removes negative feedback, resulting in hyperactivated DRN neurons (Rozeske et al., 2011).

Chronic exercise is thought to buffer against IS-induced 5-HT1AR desensitization, as basal 5-HT1AR mRNA expression is increased in physically active rats. Given that diets protected against LH behaviors and constrained IS-evoked *cfos* expression, diets may have similarly increased basal 5-HT1AR gene expression. However, no differences in basal 5-HT1AR mRNA due to diet were detected. Rather, in the mid DRN, GOS, PDX, LAC diet only significantly attenuated stress-induced 5-HT1AR mRNA downregulation. In the rostral DRN, similar trends were observed, though no significant differences were detected. The mechanisms underlying these effects are unclear; severe acute stressors are capable of downregulating 5-HT1AR mRNA (Lopez et al., 1999), and corticosteroids are thought to regulate this (Chalmers et al., 1993; Ou et al., 2003). Yet, no diet-induced differences in corticosterone were detected, and simple regressions did not demonstrate a relationship between corticosterone levels and 5-HT1AR mRNA. Future studies should investigate diet-induced modulation 5-HT1AR mRNA of known gene regulators. Taken together, these data demonstrate that the DRN is a target for diets to alter gene expression.

We also investigated *cfos* mRNA expression within DRN projection regions important for mediating LH behaviors; a subset of DRN 5-HT neurons project to the amygdala, involved in mediating exaggerated shock elicited freezing, and the dorsal striatum, involved in instrumental escape learning (Abrams et al., 2004; Christianson et al., 2010; Strong et al., 2011)). However, diet did not modulate IS-evoked *cfos* expression within these regions.

Postsynaptic 5-HT2CR mRNA expression within DRN projection regions was also explored. Downregulation of the 5-HT2CR receptor corresponds with reductions in LH behaviors (Greenwood et al., 2012b), whereas activation of this receptor contributes to LH (Christianson et al., 2010). Prior work demonstrates that exercise reduces mRNA for

postsynaptic 5-HT2CR mRNA in the striatum and amygdala (Greenwood et al., 2012b). Interestingly, we observed decreases in basal 5-HT2CR mRNA due to LAC diet only, within the dorsolateral amygdala only. This effect was more selective compared to what we previously showed due to exercise (Greenwood et al., 2012b). Nonetheless, this observation suggests that this may be a unique mechanism by which LAC diet impacts stress circuits.

Prebiotic diet could be modulating LH behaviors and gene expression in corresponding circuits during stress by impacting brain regions that control IS-evoked DRN activation. Thus, we also explored *cfos* mRNA expression in the LC (Grahn et al., 2002; Takase et al., 2005), habenula (Amat et al., 2001), and BNST (Hammack et al., 2004), brain regions capable of regulating the DRN 5-HT circuit during stress. Although IS increased *cfos* mRNA expression, we saw no attenuation of these effects due to diet. Interestingly, in the BNST, IS potentiated *cfos* mRNA expression within the GOS, PDX diet only in the lateral ventral and fusiform nuclei as well as the medial nuclei. Although it is unclear how potentiated *cfos* mRNA expression within these regions relates to stress resistance, the differential impact of these diets on subregions of the BNST suggest, again, that these diets are acting on stress circuits through differential mechanisms.

Notably, GOS, PDX and LAC diet increased basal BDNF mRNA within the cingulate and prelimbic subregions of the PFC, though this was most pronounced within the prelimbic. Others have previously shown that acute stress increases, rather than reduces, BDNF mRNA within the PFC (Bland et al., 2005). Furthermore, behavioral control-induced protection against LH depends on activation of the PFC (Amat et al., 2005), and can also increase growth factors within this brain area (Bland et al., 2007). Although the relationship between PFC growth factors and stress resistance has not been elucidated, our data demonstrate that GOS, PDX and LAC diet

can increase BDNF in the PFC, a region capable of controlling DRN activity during stress. Although we did not see any significant changes in the hippocampus, BDNF increases due to prebiotic diet are consistent with prior work, as several other studies show that prebiotics can increase BDNF; GOS intake increased hippocampal BDNF protein and NMDA receptor subunits in the PFC (Savignac et al., 2013) and similarly, neonatal supplementation of Bimuno®-galactooligosaccharides also increased hippocampal BDNF protein (Williams et al., 2016), in a manner that persisted into adulthood.

Diets did not attenuate IS-evoked activation of the HPA-axis and sympathetic nervous system, as measured by increased corticosterone and blood glucose and decreased spleen weight. Similarly, both exercise and behavioral control prevent DRN sensitization and LH behavior, but do not reduce IS-induced corticosterone responses (Greenwood et al., 2003b; Maier et al., 1986). Interestingly, simple regression did reveal that higher levels of anaerobic bacteria predicted higher blood glucose levels following IS. Certain bacteria are capable of regulating blood glucose (Mikkelsen et al., 2015), and microbiota-mediated potentiation of blood glucose following stress should be further explored.

It is possible that diets affect the CNS by altering endogenous gut microbiota. Selective fecal culture confirmed that prebiotic diets containing GOS and PDX increased Lactobacillus spp. LAC diet alone did not increase endogenous Lactobacillus spp., nor synergize with GOS, PDX to further enhance Lactobacillus spp. However, LAC diet did decrease total aerobic bacteria, perhaps due to its ability to modulate gut ecology through antimicrobial and microbiostatic activity (Alexander et al., 2012; Leon-Sicairos et al., 2006).

We also observed several notable relationships between gut bacteria and gene expression; increases in Lactobacillus spp. correlated with the degree to which diets attenuated stress-

induced increases in *cfos* mRNA and attenuated stress-evoked decreases in 5-HT1AR mRNA. In addition, higher levels of bacteria correlated with potentiated *cfos* expression following stress in certain brain regions. Complex interactions between the microbiome, gut and brain are increasingly discussed in the context of the "microbiota-gut-brain axis" (Carabotti et al., 2015; Chichlowski & Rudolph, 2015). In this bidirectional relationship, microbiota are capable of influencing the host and vice versa. Because we have no way of determining the direction of this influence, we cannot say for certain if greater increases in Lactobacillus spp. or aerobic/ anaerobic bacteria produced greater adaptations in gene expression. It is also important to note that diets may have affected gut ecology in ways that could not be assessed using culture. Thus, the lack of more global assessment tools, such as 16S rRNA analysis, and non-culture methods is a limitation in the current study.

In future studies investigating the mechanisms underlying bacteria to DRN communication, the vagus nerve should be explored. Bacteria may interact with receptors on vagal afferents originating in the cecum (Altschuler et al., 1991), which ascend to the nodose ganglion and project to the NTS. The NTS then projects to the LC, which projects to the DRN. Through this pathway, vagal nerve stimulation can influence the 5-HT system (Manta et al., 2009). While diets did not alter basal *cfos* mRNA expression within the NTS, diets containing GOS and PDX did indeed increase basal *cfos* mRNA within the DRN, an NTS projection site. These results thus do not preclude the possibility that these prebiotic diets are capable of influencing the brain through this pathway, and further studies are needed.

Short chain fatty acid (SCFA) production should also be explored in future studies; it is possible that microbial fermentation of prebiotic fibers into SCFAs play a role in conferring diet effects on the brain. Notably, butyrate, an SCFA capable of immune modulation and histone deacetylase

inhibition, can induce positive changes in brain plasticity and function, as well as mood and behavior (Stilling et al., 2016). More research is needed to investigate whether microbially derived butyrate is capable of altering gene expression within stress-sensitive circuits.

It is also possible that these diets impact central stress circuits through a mechanism independent from the gut microbiota. For instance, a LAC receptor is present in the choroid plexus, through which LAC is transferred into the cerebrospinal fluid (Kamemori et al., 2008; Talukder et al., 2003). In addition, Huang and colleagues (Huang et al., 2007) demonstrated LAC receptors on the surface of brain endothelial capillary cells and homogenized brain tissue, while a recent study demonstrated that LAC enhanced learning and upregulated BDNF as well as signaling pathways involved in neuroplasticity (Chen et al., 2015). Collectively, these studies suggest that LAC can directly impact the brain.

Taken together, the present data demonstrate that GOS, PDX, and LAC attenuate IS-induced LH behaviors, and alter gene expression in corresponding circuits. These changes are unique to each diet, and the mechanisms by which these diets alter stress circuits warrants further exploration. Nonetheless, this work offers preclinical evidence supporting a role for prebiotic dietary ingredients in promoting stress resistance.

vi. References

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CHAPTER IV

EXERCISE AND PREBIOTICS PRODUCE STRESS RESISTANCE: CONVERGING IMPACTS ON STRESS-PROTECTIVE AND BUTYRATE-PRODUCING GUT BACTERIA

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i. Abstract

The gut microbial ecosystem can mediate the negative health impacts of stress on the host. Stressor-induced disruptions in microbial ecology (dysbiosis) can lead to maladaptive health effects, while certain probiotic organisms and their metabolites can protect against these negative impacts. Prebiotic diets and exercise are feasible and cost- effective strategies that can increase stress-protective bacteria and produce resistance against the detrimental behavioral and neurobiological impacts of stress. The goal of this review is to describe research demonstrating that both prebiotic diets and exercise produce adaptations in gut ecology and the brain that arm the organism against inescapable stress-induced learned helplessness. The results of this research support the novel hypothesis that some of the stress-protective effects of prebiotics and exercise are due to increases in stress-protective gut microbial species and their metabolites. In addition, new evidence also suggests that prebiotic diet or exercise interventions are most effective if given early in life (juvenile–adolescence) when both the gut microbial ecosystem and the brain are plastic. Based on our new understanding of the mechanistic convergence of these interventions, it is feasible to propose that in adults, both interventions delivered in combination may elevate their efficacy to promote a stress-resistant phenotype.

ii. Stress and health

a. Introduction

The majority of health-care professionals and stress physiologists agree that repeated, continuous, and excessive stressor exposure adversely affects both mental and physical health. The biological response initiated by stressor exposure is a highly conserved cascade of events orchestrated by the sympathetic nervous system and hypothalamic–pituitary–adrenal (HPA) axis in efforts to arm the organism with the metabolic substrates needed for survival; coordinated increases in energy mobilization, heart rate, respiration, and immunity serve to prepare the organism to either successfully fight or flee an impeding threat. While these survival-promoting aspects of the stress response are considered adaptive, excessive or prolonged stimulation of the stress response produces maladaptive health consequences that persist long after the stressor subsides. These widespread negative health impacts are well documented and include increased vulnerability to mental health disorders, such as anxiety disorders and depression (Cohen et al., 2007; Grippo & Johnson, 2009), and increased susceptibility to illness due to pathogens (Padgett & Glaser, 2003; Sheridan et al., 1998), neoplastic cellular replication (e.g., cancer; Moreno-Smith, Lutgendorf, & Sood, 2010), cardiovascular disease (Iso et al., 2002), as well as inflammatory disease (Reber et al., 2011). Given that exposure to stressors is often an inevitable part of life, avoiding stress is not an effective means to maintain health. Identifying factors that can mitigate the undesirable consequences of stress and promote a stress-resistant phenotype is therefore an important goal for stress researchers.

The degree to which stressor exposure impacts our health depends on a variety of factors. Some of these factors are outside of our control; these may include the nature of the stressor (such as intensity, controllability, and chronicity) as well as predetermined biological features

(such as age, gender, and genes). For instance, the mental health impacts of stressors experienced earlier in life can be longer lasting and more severe compared with the effects of stressors experienced in adulthood. Indeed, epidemiological and clinical literature in humans demonstrates that adverse experiences occurring earlier in life are major risk factors for psychiatric disorders in adulthood (Culpin et al., 2015; Heim & Nemeroff, 2001; Heim et al., 2004; Whitesell et al., 2009), including depression, anxiety, illicit drug use, stunted learning, and attention disorders. Environmental factors we are capable of controlling, such as physical fitness, social support, and diet, can also regulate the degree to which stressor exposure impacts health. These variables are of special interest to researchers, as they represent feasible, cost-effective strategies that can promote stress robustness. Identifying such strategies, and exploring the mechanism by which they mitigate the health consequences of stress can lead to effective treatments and interventions for stress-related disorders.

iii. Gut microbial organisms and their metabolites are emerging mediators of the health impacts of stress

a. Stress disrupts health by disturbing gut microbes

Commensal gut bacterial symbiosis (composition and balance) plays an important role in many aspects of physiology and health. It is now well understood that gut microbes maintain and influence key aspects of host physiology, including immune development and function (Hrncir et al., 2008; Tlaskalova-Hogenova et al., 2004), energy metabolism (Geurts et al., 2014; Ridaura et al., 2013; Turnbaugh et al., 2006), nutrient production (Conly et al., 1994; Hill, 1997; Sommer & Backhed, 2013), gastrointestinal barrier integrity (Berg & Garlington, 1979), circadian rhythms (Leone et al., 2015), as well as brain function and behavior (G. Clarke et al., 2013; Desbonnet et al., 2014; Diaz Heijtz et al., 2011; Naseribafrouei et al., 2014; Tarr et al., 2015). Stressor

exposure can damage this dynamic ecology of intestinal microbes by producing dysbiosis. Dysbiosis is implicated in the pathogenesis of a wide variety of disease states, including obesity (Ley et al., 2005; Ridaura et al., 2013), kidney disease (Nallu et al., 2016), cancer (Moore & Moore, 1995), and psychiatric disorders (Desbonnet et al., 2014; Yarandi et al., 2016), to name a few. Given that the adverse health consequences of stress overlap with those that are produced by gut microbial dysbiosis, stress-induced disruptions in gut ecology constitute a promising potential mechanism by which stress disrupts health. Indeed, gut microbes contribute to many of the physiological and behavioral consequences of stressor exposure, including immune modulation (Bailey, 2012; Bailey et al., 2011; Maslanik et al., 2012), disruptions in intestinal permeability and bacterial translocation (Ait-Belgnaoui et al., 2012; Eutamene et al., 2007; Zareie et al., 2006), altered social and emotional behavior (Bailey & Coe, 1999), impaired memory (Gareau et al., 2011), and HPA axis dysregulation (Gareau et al., 2007; Sudo et al., 2004). Stressor-induced disruptions in the gut microbial ecosystem have thus emerged as a means by which stress disrupts health.

b. Stress-protective microbes: probiotic bacteria

Recognizing the significant role of the gut microbial ecology as a mediator of stress-evoked adverse health consequences has exposed its promise as a target for intervention. If stress-induced disruptions in gut microbial ecology can produce and potentiate disease, then perhaps maintaining the integrity of this ecosystem can protect host health in the face of stress. Indeed, certain probiotic bacterial species, largely consisting of lactic acid-producing bacteria (Lutgendorff et al., 2008), have been shown to mitigate some of the maladaptive health consequences of stressor exposure. For instance, *Lactobacillus farciminis* strengthened intestinal barrier integrity in the face of water-avoidance stress (Da Silva et al., 2014), while combined

administration of *Lactobacillus acidophilus* and *Bifidobacterium longum* attenuated stress-induced gastrointestinal symptoms in humans (Diop et al., 2008). A large extent of this literature also demonstrates the effectiveness of probiotic bacteria in lessening the impact of stress on the central nervous system. Treatment with *Bifidobacteria infantis* normalized stress-evoked behavioral deficits in the forced swim test as well as accompanying immune and neurotransmitter perturbations (Desbonnet et al., 2010); *B. longum* and *Bifidobacterium breve* differentially attenuated stress-induced anxiety (Savignac et al., 2014). *Lactobacillus helveticus* and *B. longum,* in combination, prevented stress-induced decreases in hippocampal neurogenesis (Ait-Belgnaoui et al., 2014). *L. farciminis* was capable of normalizing HPA responses (Ait-Belgnaoui et al., 2012), and comparably, *Lactobacillus rhamnosus* diminished stress-evoked anxiety- and depressive-like symptoms and HPA responses (Bravo et al., 2011). Along the same lines, *Lactobacillus plantarum* diminished depressive-like behavior, HPA responses, and proinflammatory cytokine profiles following early-life stressor exposure (Liu et al., 2016).

c. Stress-protective microbes: butyrate-producing bacteria

The fermentation products of certain microorganisms produce a wide variety of health benefits. Selective bacterial species are capable of producing short-chain fatty acids (SCFAs) through fermentation of complex carbohydrates. SCFAs, consisting of acetate, propionate, and butyrate produced in a ratio of 60:20:20, respectively, have been implicated as a primary mechanism by which certain microbial organisms affect host physiology.

Notably, butyrate was recently shown to produce beneficial adaptations in brain plasticity and function. Butyrate is a potent histone deacetylase (HDAC) inhibitor both in vitro and in vivo (Boffa et al., 1978; Candido et al., 1978; Riggs et al., 1977; Sealy & Chalkley, 1978), meaning that it can facilitate acetylation of histone proteins and promote gene transcription by attenuating

the acetyl group turnover rate (Reeves & Candido, 1978; Sealy & Chalkley, 1978). One study demonstrated that an IP dose of butyrate can increase brain-derived neurotrophic factor (BDNF) promoter acetylation, enhance BDNF transcription within the hippocampus, and promote hippocampal-dependent learning (Intlekofer et al., 2013). Beyond hippocampal function, other work demonstrated that butyrate can produce antidepressant-like effects (Schroeder et al., 2007). Recently, in a rodent model, butyrate administered IP attenuated chronic stress-induced depression, as measured by sucrose preference, light/dark test, forced swim test, and tail suspension, as well as attenuated stress-induced decreases in hippocampal immediate-early gene expression, histone acetylation, and BDNF protein expression (Han et al., 2014). Although it is still unclear whether endogenous butyrate, produced by intestinal microbes, is capable of interacting with the brain, this SCFA serves as a notable potential mechanism by which certain microbes alter brain and behavior. This body of work collectively demonstrates that promoting gut microbial ecology by increasing probiotic and butyrate- producing microorganisms may protect the host from many the negative impacts of stress on neurobiology and behavior.

iv. Prebiotic diets and exercise can promote stress-protective probiotic bacteria

a. Prebiotics promote the growth and function of probiotic and butyrate producing bacteria

Ingesting *pre*biotic dietary fiber is a feasible and effective means by which to increase probiotic bacterial species. Prebiotic dietary ingredients constitute a class of compounds that are inadequately metabolized by the host, and instead travel to the lower GI tract where they undergo fermentation by gut microbes that are armed with the enzymatic machinery to utilize prebiotic fibers as sources of fuel. Interactions between prebiotics and gut microbes support the growth and function of existing probiotic bacterial species residing within the colon (Bouhnik et al., 1997; Cardelle-Cobas et al., 2011; Herfel et al., 2011; Moro et al., 2002; Schwab & Ganzle,

2011), often resulting in the proliferation of probiotic bacterial species and enhancements in host health (Roberfroid, 2007; Roberfroid et al., 2010). Indeed, a variety of synthetic and naturally occurring compounds have been described as having prebiotic capabilities, ranging from polysaccharides, oligosaccharides, and polyols (Ouwehand et al., 2005; Roberfroid et al., 2010). Importantly, prebiotics may offer several benefits to probiotics, including the ability to produce broader and potentially more stable changes in microbial ecology through the expansion of numerous probiotic species.

A variety of prebiotics have been shown to enhance the growth and activity of stress-protective microbial organisms. For instance, the synthetic soluble fiber polydextrose (PDX) successfully increased stress-protective lactic acid-producing bacteria *Lactobacillus* spp. in piglets (Herfel et al., 2011), and the polyol disaccharide lactitol and xylooligosaccharides promoted the growth of *Lactobacillus* and *Bifidobacteria* spp. in culture (Makelainen et al., 2010). PDX and soluble corn fiber favorably shifted gut microbial composition in a group of healthy adults; soluble corn fiber increased *Lactobacillus* spp., and both successfully increased bacteria with anti-inflammatory properties (Hooda et al., 2012). Similarly, healthy adults fed the synthetic soluble fiber galactooligosaccharide (GOS) exhibited increases in *Bifidobacteria* spp. (Davis et al., 2010).

Due to their resistance to digestion in upper GI tract, prebiotics also play a significant role in increasing SCFA production by serving as substrates for colonic fermentation (Macfarlane et al., 2006). For instance, chemically modified resistance starches were capable of modulating concentrations of all three SCFAs. Fructooligosaccharides have been shown to increase butyrate in humans (Vitali et al., 2012). Similarly, arabinoxylans and arabinoxylans oligosaccharides, types of dietary fibers found in wheat (Damen et al., 2011), increased butyrate

and butyrate-producing bacteria, while inulin (Jung et al., 2015) was also capable of increasing butyrate levels. Prebiotic dietary ingredients therefore present a promising tool by which to expand stress-protective microbial species (lactic acid and butyrate-producing bacteria) and protect the host from the behavioral, neurobiological, and physiological effects of stress.

b. Exercise promotes the growth and function of probiotic and butyrate producing bacteria

Researchers are also realizing that environmental and lifestyle factors known to promote stress resistance may do so by adaptively modulating the gut microbiota. Exercise is one such factor that can both produce stress resistance and modulate the gut microbiota. Recently, it has become clear that exercise is also capable of producing adaptations within gut microbial ecology in both rodents and humans (Allen et al., 2015; Campbell et al., 2016; S. F. Clarke et al., 2014; Denou et al., 2016; Evans et al., 2014; Kang et al., 2014; Matsumoto et al., 2008; Petriz et al., 2014; Welly et al., 2016), including promoting the growth of probiotic bacterial species such as *Bifidobacteria* (Lambert et al., 2015) and *Lactobacillus* spp. (Mika & Fleshner, 2016; Queipo-Ortuno et al., 2013) as well as butyrate-producing bacteria (Matsumoto et al., 2008; Mika & Fleshner, 2016). Given that exercise produces increases in stress-protective bacteria, it is possible that exercise-induced increases in these bacteria are mechanistically involved in the stress-protective effects of exercise. Indeed, we as well as others have postulated that exercise-induced changes in gut bacterial species contribute to the health-enhancing effects of exercise, including the ability of exercise to exert positive effects on mood and behavior (Cerda et al., 2016; Kang et al., 2014; Mika & Fleshner, 2016; Yuan et al., 2015).

v. Prebiotic diets and exercise promote resistance against the behavioral and neurobiological consequences of inescapable stress through unique and overlapping mechanisms

In the remainder of this chapter, we discuss our research demonstrating that both prebiotic diets and exercise are capable of promoting resistance against the anxiety- and depressive-like behaviors produced by inescapable stress (IS). Given that the neurobiological mechanisms by which IS produces anxiety- and depressive-like behaviors are well known, this stress paradigm can be very useful for investigating the mechanisms by which novel treatments, like prebiotics, can protect against the negative mental health effects of excessive stress. Using this paradigm, we discuss our findings demonstrating that both exercise and prebiotic diet can alter gene expression within the brain's serotonergic circuits that subserve IS-induced anxiety- and depressive-like behaviors. We will discuss the potential mechanisms by which gut bacteria and their metabolites influence these neurobiological adaptations. We will discuss the importance of age, specifically how these interventions may be more successful earlier in life due to the inherent plasticity of the central nervous system as well as gut microbial ecology and lastly, the possibility of utilizing these interventions in tandem to promote more robust and lasting adaptive changes in adult organisms.

a. Prebiotics positively impact brain and behavior

There has recently been increasing interest in investigating the impact of prebiotics on the CNS and behavior. Though this literature is still in its infancy, early evidence suggests that prebiotic dietary ingredients are indeed capable of modulating central BDNF among other plasticity-related proteins, neurotransmitters, cytokines, as well as anxiety, and emotional processing (Savignac et al., 2013, 2016; Schmidt et al., 2015; Williams et al., 2016). Preliminary evidence also suggests that prebiotic diets are capable of protecting the host against the negative mental health effects of stress. Prebiotics derived from human milk were recently shown to successfully attenuate stress-induced anxiety-like symptoms in a battery of tests (Tarr et al.,

2015). There is thus compelling evidence that prebiotics are capable of protecting against the negative mental health consequences of stress, though the neural mechanisms by which this occurs are unclear.

b. Prebiotics protect against IS-induced stress resistance

We have recently demonstrated that ingestion of diets containing the synthetic, soluble prebiotics GOS and PDX as well as the naturally occurring iron-binding glycoprotein lactoferrin (LAC) for 4 weeks attenuated the behavioral and neurological consequences of IS (Mika et al., 2016, in review; Rumian et al., 2014). IS, consisting of a series of uncontrollable and unpredictable local shocks over a 2 h period, leads to the development of a series of behaviors that arguably represent symptoms of human stress-related psychiatric disorders, such as depression and anxiety (Greenwood & Fleshner, 2008). The depressive and anxiety-like behaviors produced by exposure to IS are collectively termed learned helplessness (LH), and include exaggerated shock-elicited fear, a measure of anxiety-like behavior, and instrumental escape learning deficits, a measure of a cognitive deficit associated with depression. Three dietary formulations, consisting of GOS and PDX, LAC alone, and GOS, PDX with LAC, all attenuated IS-induced LH.

Furthermore, each diet produced distinct adaptations in gene expression within central stress circuits regulating the development of these LH behaviors. The neurobiological mechanisms by which IS alters 5-HT circuits and produces LH behaviors have been demonstrated by Maier and Watkins (2005). Exposure to IS hyperactivates dorsal raphe nucleus (DRN) 5-HT neurons and desensitizes the inhibitory 5-HT1A autoreceptor (5-HT1AR) on 5-HT neurons within the DRN. Since activation of this presynaptic Gi/Go-coupled autoreceptor reduces 5-HT synthesis and neuronal firing (Chen & Penington, 1996; Valdizan et al., 2010), 5-

HT1AR serves as an important negative feedback mechanism that guards against excessive 5-HT release. However, when IS-induced activation of DRN neurons desensitizes 5-HT1AR (Rozeske et al., 2011), this negative feedback signal is rendered temporarily inactive, and DRN 5-HT neurons become temporarily sensitized. Sensitized DRN neurons then release copious amounts of 5-HT in response to even mild stimuli, such as those experienced during subsequent behavior testing. Excessive release of 5-HT in downstream brain structures (for review, see Abrams et al., 2004) such as the amygdala and the dorsal striatum (for reviews, see (Graeff et al., 1996; Lowry et al., 2005)) produces deficits in shock-elicited fear and instrumental escapes learning, respectively (Amat et al., 1998a, 1998b).

Although each diet was capable of protecting against IS-induced LH behaviors, they produced both overlapping and unique neurobiological adaptations within 5-HT circuits. All diets decreased IS-induced *cfos* mRNA within DRN neurons, indicating that these diets may be capable of producing neurobiological adaptations within DRN 5-HT circuits that constrained DRN activity during stress. Interestingly, we also observed an increase in *cfos* mRNA expression in home cage rats (rats not exposed to IS) following diets containing GOS and PDX, perhaps indicating that diets containing this particular blend of prebiotics are capable of impacting baseline DRN neuronal activity. In addition, diets containing all three ingredients (GOS, PDX, and LAC) attenuated stress-evoked decreases in 5-HT1AR mRNA, and increased BDNF mRNA within the prefrontal cortex. These results demonstrate that these blends of prebiotics can protect against IS-induced LH, perhaps by producing neuroplastic adaptations that constrain 5-HT DRN neurons in the face of IS. The mechanisms by which this occurs may depend on the structure and function of each unique prebiotic ingredient.

c. Exercise positively impacts brain and behavior

In contrast to the impact of prebiotics on neurobiology and behavior, the stress-protective effects of exercise have been well established. Human and rodent studies demonstrate that regular, daily physical activity can protect against the development of stress-related psychiatric disorders, such as anxiety and depression. Clinical and epidemiological studies report that regular exercise can improve symptoms associated with depression and anxiety (Carek et al., 2011; Mason & Powell, 1985; Mortazavi et al., 2012; Paluska & Schwenk, 2000) whereas physical inactivity is associated with increased incidence of psychological disorders (Hamer & Stamatakis, 2014).

d. Exercise protects against IS-induced LH

Using rodent models, we have repeatedly demonstrated that 6 weeks of exercise in adulthood can prevent LH behaviors after IS exposure, demonstrating that physical activity is a poweful tool that can protect the organism against the debilitating effects of stress (Greenwood & Fleshner, 2008; Greenwood et al., 2005; Greenwood et al., 2003; Greenwood et al., 2012a).

We have also previously characterized a number of neurobiological mechanisms by which exercise can protect against the behavioral consequences of IS. Our lab has demonstrated that 6 weeks of exercise in adult rats can increase 5-HT1AR mRNA in the dorsal aspect of the rostral and mid DRN (Greenwood et al., 2003; Loughridge et al., 2013). Exercise-induced increases in 5-HT1AR mRNA can protect against IS-induced 5-HT sensitization through a variety of mechanisms (reviewed in Greenwood & Fleshner, 2011). Evidence from prior studies demonstrates that 6 weeks of physical activity can attenuate the stress-induced activation of 5-HT DRN neurons (Greenwood et al., 2003), suggesting that an increase in 5-HT1AR number may perhaps maintain negative feedback in the face of stress. Recent work supports this hypothesis; using in vivo microdialysis, Clark et al. (2015) reported that 6 weeks of wheel

running prior to IS prevented the exaggerated release of 5-HT in the dorsal striatum evoked by mild stress 24 h later. Collectively, this work has shown that exercise can produce adaptations that protect against the negative behavioral and neurobiological consequences of IS-induced exaggerated 5-HT release.

e. Prebiotics and exercise produce stress resistance via unique neuroplastic changes

As we have just begun to investigate the neurobiological adaptations produced by prebiotics, the neural mechanisms by which exercise protects against LH are better characterized in comparison to the mechanisms by which prebiotics protect against LH. However, from our work to date, we can conclude that exercise and prebiotic diets both produce unique, neuroplastic changes within 5-HT circuits that result in constrained DRN activity during stress. For instance, exercise has repeatedly produced an increase in 5-HT1AR mRNA, whereas prebiotic diet did not. Exercise produces a wide variety of neurobiological adaptations that are likely independent of exercise-induced adaptations within gut microbial organisms. Thus, one would expect the neurobiological adaptations produced by exercise to be more extensive. Nonetheless, despite these differential neuroplastic changes, both manipulations alter gene expression within serotonergic circuits and produce similar behavioral effects as well as similar adaptations in microbial ecology.

vi. The stress protective effects of prebiotics and exercise may be age dependent

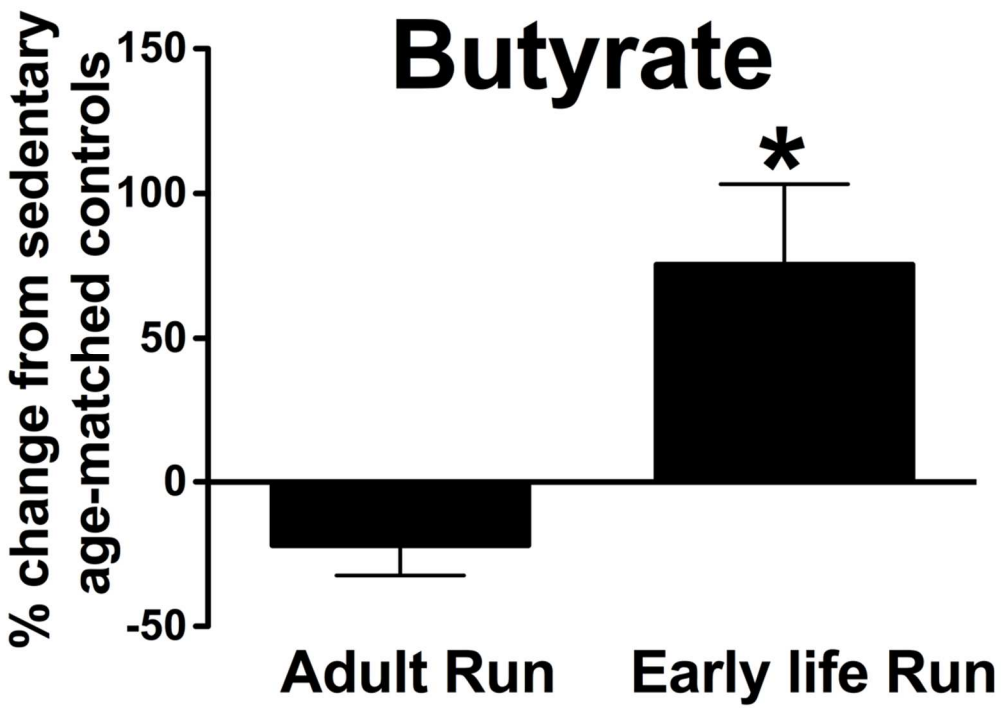
a. Age-dependent effects of exercise and prebiotic diet on stress protective bacteria and butyrate

Prebiotic diets and exercise can improve health throughout the life span, and the adaptations in gut bacteria as well as the central nervous system and behavior produced by exercise training and prebiotics are well documented at all ages. However, it is important to note that our observations demonstrating that prebiotic diets containing GOS, PDX, and LAC can

increase stress-protective bacteria were completed in young rats; diets were initiated at postnatal day 24 (Mika et al., 2016, in review). Although others have demonstrated that increases in endogenous probiotic bacteria are possible in adults following GOS and PDX, it is unclear whether the tested prebiotic dietary formulations would have produced similar changes within the same time course.

On the other hand, we have demonstrated that exercise-induced changes in gut microbiota are more robust in early life. We have recently compared the influence of early vs late-life exercise on gut microbial ecology and demonstrated that 6 weeks of exercise initiated in early life have a greater impact on the gut microbiota than exercise initiated in adulthood (Mika et al., 2015). Importantly, exercise begun earlier in life increased *Lactobacillus* spp. and bacterial genera that are capable of producing butyrate (Mika & Fleshner, 2016), while on the other hand, adult-onset exercise modestly impacted the gut microbiota and failed to produce alterations in stress- protective bacteria. Similarly, 3 weeks of exercise initiated earlier in life, but not exercise initiated in adulthood, are capable of increasing fecal butyrate concentrations (Fig. 1). Thus, exercise earlier in life more effectively produces a stress robust microbial ecosystem by increasing probiotic bacteria, butyrate-producing bacteria, as well as butyrate production.

Figure 1



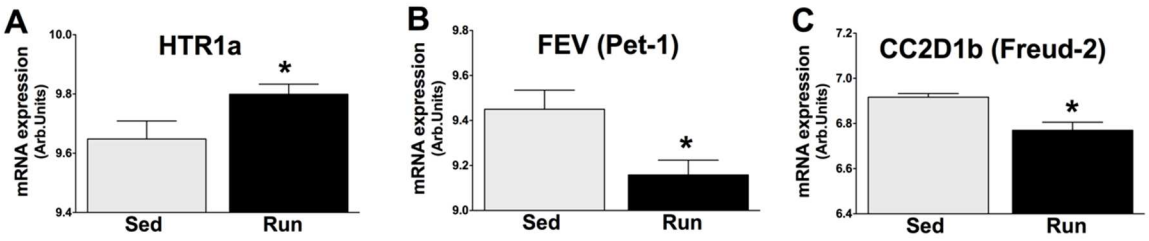
Percent change in butyrate levels of runners from their respective age matched sedentary controls. Briefly, rats were given access to voluntary running wheels or remained sedentary for 3 weeks. Fecal samples were collected at the end of the 3 weeks and sent to the Metabolomics Core of the University of Michigan for short chain fatty acid analysis, and processed in accordance to previous protocols (Chassaing et al., 2015). ANOVA shows there is a significant increase in percent change of butyrate levels in the rats that began running in early life (postnatal day 24 at the start of exercise) as compared to adult runners (postnatal day 70 at the start of exercise; ($F(1,8)=0.0064$, $p=0.0064$). *$p < 0.05$.

b. Age-dependent effects of exercise and prebiotic diet on neurobiology and behavior

Because our observations demonstrating that prebiotic diets can protect against IS-induced LH behaviors were completed in young rats, we cannot be sure if similar effects are possible in adults. We have, however, compared the neurobiological and behavioral effects of exercise initiated in early life with those produced by adult-onset exercise. We have demonstrated that exercise initiated during early, critical periods in development can produce behavioral protection against LH behaviors that is more persistent following cessation of exercise, compared with exercise initiated in adulthood (Mika, Bouchet, Spence, Greenwood, & Fleshner, 2013). Preliminary data also demonstrate that exercise in early life can produce long-lasting increases in 5-HT1AR mRNA. In comparison, increases in 5-HT1AR mRNA were transient in adults.

Interestingly, early-onset exercise can also impact a wide variety of genes within DRN 5-HT neurons that may be necessary for conferring stress resistance. Using laser capture microdissection of the DRN and subsequent Affymetrix microarray for the investigation of gene expression within the DRN neurons, we have also demonstrated that exercise initiated earlier in life (during adolescence, prior to adulthood) can produce unique changes in gene expression for genes involved in regulating 5-HT1A as well as those involved in producing epigenetic changes within the DRN. As demonstrated in Loughridge et al. (2013), mRNA expression levels of HTR1A, the gene responsible for 5-HT1AR expression, increased in adolescent runners compared to their sedentary counterparts (Fig. 2A). On the other hand, exercise decreased mRNA expression level of both FEV and CC2D1b, known transcriptional regulators capable of repressing HTR1A (Le Francois et al., 2008; Albert et al., 2011).

Figure 2



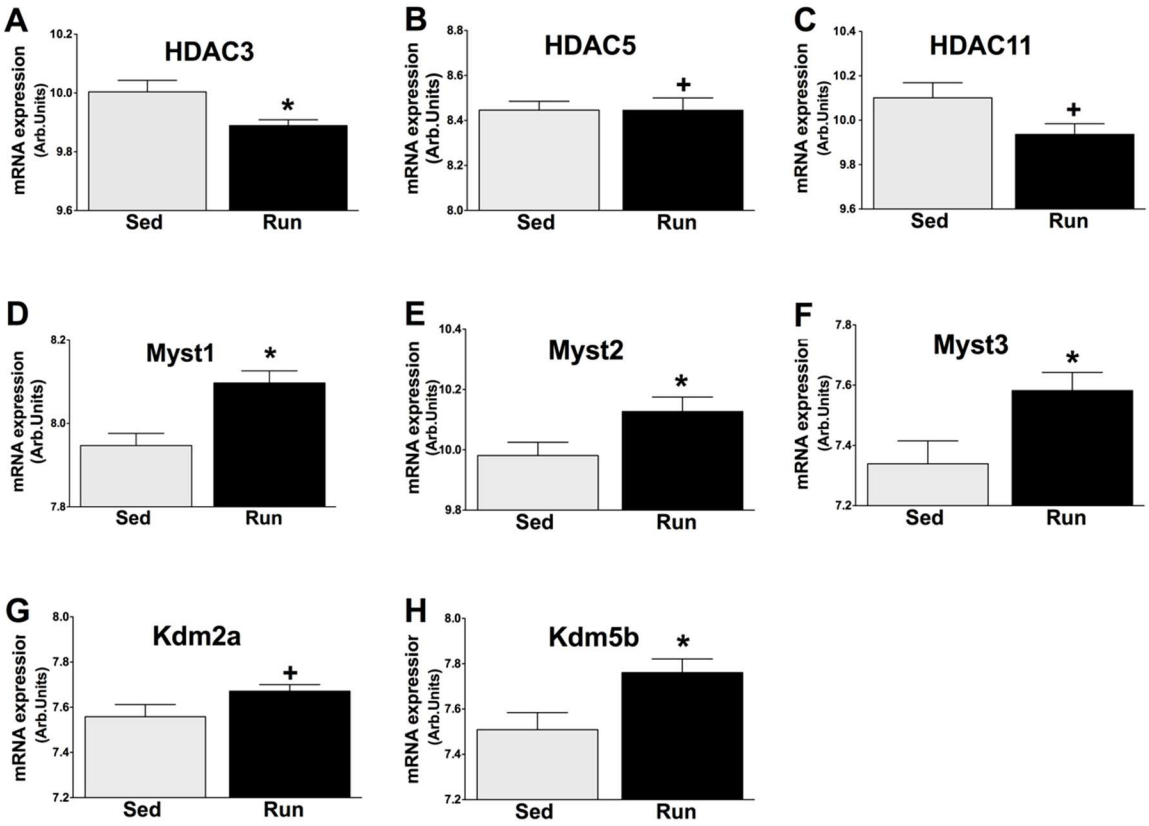
mRNA expression levels of genes relevant to 5-Hydroxytryptamine (serotonin) receptor 1A (HTR1a) gene expression within the DRN, determined by microarray analysis (Loughridge et al., 2013). Briefly, adolescent rats were given access to a voluntary running wheel or remained sedentary for 6 weeks, and were subsequently sacrificed and brains extracted for analysis of gene expression (Loughridge et al., 2013). Briefly, laser capture microdissection of the DRN allowed for the analysis of gene expression within DRN neurons only, and mRNA expression was measured using Affymetrix microarray. As depicted in Loughridge et al (Loughridge et al., 2013), mRNA expression levels of HTR1a increased ($F_{1,12}=5.353$, $p=0.0392$; A) in runners as compared to their sedentary counterparts, whereas there was a decrease in mRNA expression level of both FEV ($F_{1,12}=7.649$, $p=0.0171$; B) and CC2D1b ($F_{1,12}=11.035$, $p=0.0061$; C) in runners as compared to sedentary controls. All data were analyzed by ANOVA, $^*p < 0.05$ as compared to sedentary group.

Fig. 3 depicts early-life exercise-induced changes in gene expression for genes that are implicated in epigenetic regulation; exercise altered gene expression for a variety of these epigenetic regulators. Specifically, exercise significantly decreased mRNA expression levels of HDAC3 and produced trends toward decreases in HDAC5 and HDAC11. HDACs remove an acetyl group from acetylated histone proteins and typically lead to a decrease in transcription of the associated DNA. Studies show these HDACs are each capable of reducing transcription of various genes (Lewandowski et al., 2015; Zhao et al., 2016; Wein et al., 2015; Chen et al., 2016a). In contrast to the mechanism by which HDACs regulate gene expression, histone acetyltransferases (HATs) add an acetyl group to histone proteins, which typically results in enhanced gene transcription of associated genes. Fig. 3D–F consists of HATs; exercise increased mRNA expression levels of Myst1, Myst2, and Myst3, respectively. Myst1, Myst2, and Myst3 have been shown to acetylate certain lysine residues on various histone proteins (Chen et al., 2014; Kim et al., 2015; Mishima et al., 2011; Sheikh et al., 2015; Zhu et al., 2015) and are capable of increasing expression of their associated genes. Collectively, these results demonstrate that exercise orchestrates epigenetic changes that lead to increases in gene expression within the DRN. This pattern is continued in Fig. 3G, where exercise produces a trend toward an increase in Kdm2a mRNA expression; Kdm2a acts as lysine demethylase to enhance transcription (Chen et al., 2016b). On the other hand, in Fig. 3H, exercise increases Kdm5b, which has been shown to repress gene transcription (Yamane et al., 2007).

Because we only measured global mRNA expression of DRN-specific genes involved in epigenetic modifications, we cannot say for certain what genes these epigenetic regulators are acting on, nor whether early-life exercise actually produced epigenetic modifications. However, these data imply that epigenetic modifications in the DRN following early-life exercise are

possible and could potentially explain the longer-lasting increases in 5-HT1AR mRNA that are also observed following exercise in early life.

Figure 3



mRNA expression levels of genes related to epigenetic processes within the DRN, determined by microarray analysis. Briefly, adolescent rats were given access to a voluntary running wheel or remained sedentary for 6 weeks, and were subsequently sacrificed and brains extracted for analysis of gene expression (Loughridge et al., 2013). Briefly, laser capture microdissection of the DRN allowed for the analysis of gene expression within DRN neurons only, and mRNA expression was measure using Affymetrix microarray. Data is grouped based on function. Exercise decreased mRNA expression levels of HDAC3 ($F_{1,12}=8.087$, $p=0.0148$; A), HDAC5 ($F_{1,12}=4.178$, $p=0.0635$; B), and HDAC11 ($F_{1,12}=4.151$, $p=0.0643$; C). Exercise increased mRNA expression levels of Myst1 ($F_{1,12}=8.425$, $p=0.0133$; D), Myst2 ($F_{1,12}=4.682$, $p=0.0514$; E), and Myst3 ($F_{1,12}=6.567$, $p=0.0249$; F). Additionally, exercise increased mRNA expression levels of Kdm2a ($F_{1,12}=3.99$, $p=0.06990$; G) and Kdm5b ($F_{1,12}=7.096$, $p=0.0206$; H). All data were analyzed by ANOVA, *$p<0.05$ as compared to sedentary group; + $p<0.07$ as compared to sedentary group.

c. Early-life increases in stress protective bacteria can promote robust and lasting stress resistance

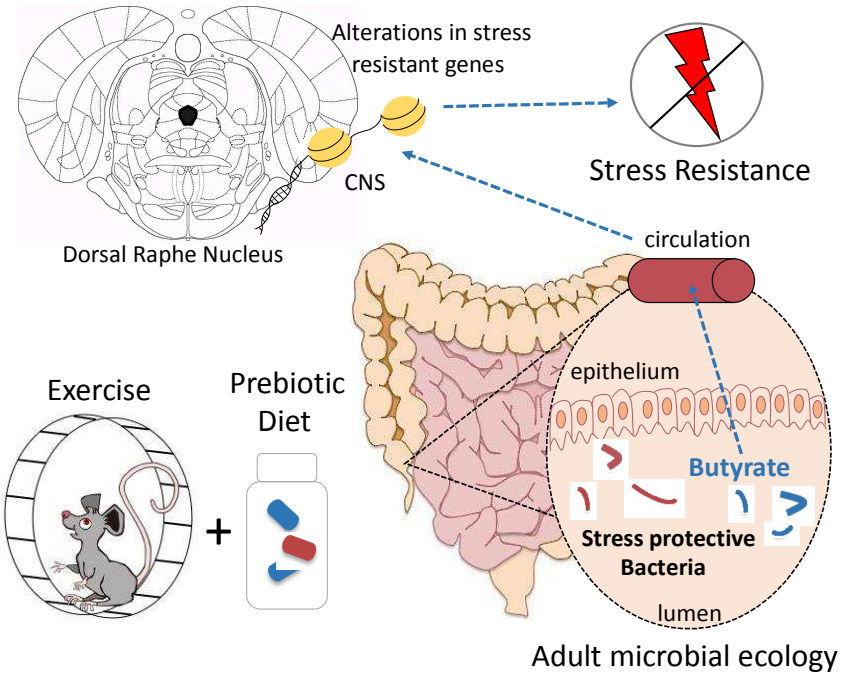
We have discussed our observations demonstrating that exercise initiated during early, sensitive periods can produce persistent adaptations in brain function and gut microbial ecology, two seemingly separate physiological systems of the developing host. We have demonstrated that early-life exercise increases stress-protective and butyrate-producing microbes, and is capable of producing long-lasting stress resistance and changes in gene expression for 5-HT1AR and epigenetic regulators. Although we have not demonstrated that these observations are causally related, it is possible that early-life exercise-induced increases in butyrate production communicate with the brain to alter and maintain gene expression for genes that are necessary for producing longer-lasting stress resistance.

d. Potential synergy between probiotics and exercise: hope for adults

These data beg the question of whether it is possible to achieve lasting and robust stress resistance in adult organisms. If some of the stress-protective effects of exercise are produced by early-life exercise-induced changes in gut microbes, then perhaps it would be possible to recapitulate some of these effects in adults by producing more robust increases in stress-protective bacteria. In adulthood, the microbial ecosystem is less receptive to change. Gut microbial ecology demonstrates a greater susceptibility for change in early life, perhaps partially because microbial diversity progressively increases with age (Yatsunenko et al., 2012). Furthermore, the gut ecology of younger children (Ringel-Kulka et al., 2013) and juvenile rats (Mika et al., 2015) is more plastic, variable, and less stable than adults. The plasticity and lack of diversity characteristic of an immature gut suggests that it may be more sensitive to manipulation (Ringel-Kulka et al., 2013), while the increase in diversity as organisms age eventually renders

the adult gut less susceptible. We have previously shown that exercise alone in adults is not capable of increasing stress-protective probiotic and butyrate-producing bacteria, but perhaps exercise in tandem with a prebiotic diet will synergistically enhance these bacteria in adults (Fig. 4). Furthermore, if both manipulations could simultaneously evoke greater microbial change, it would be prudent to investigate whether these changes would lead to more robust and lasting protection against the negative mental health effects of stress. Future research should investigate this possibility, as it may provide a feasible and cost-effective means by which previously sedentary adults can increase stress robustness.

Figure 4



Potential synergistic effects of exercise and prebiotic diet. Combining exercise and prebiotic diet can increase the amount of stress protective bacteria within the gut. Butyrate produced by these bacteria can enter the circulation and cross the blood-brain barrier to induce epigenetic changes that modify and maintain genes involved in promoting stress resistance within the DRN, thus potentially helping to produce longer lasting and more robust stress resistance.

vi. References

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CHAPTER V

GUT BACTERIA MAY BE NECESSARY FOR EARLY LIFE EXERCISE-INDUCED STRESS RESISTANCE: A WORK IN PROGRESS

i. Abstract

In early life, positive lifestyle choices such as exercise have the opportunity to produce robust and enduring adaptations in multiple systems. In rodents, we have shown that exercise initiated during the juvenile period is capable of producing more enduring protection against depressive-like behavior following stressor exposure than exercise in adulthood. We have also demonstrated that juvenile onset exercise has a greater impact on gut microbial ecology than adult exercise, and increases probiotic gut bacterial species. The immature brain may be more receptive to microbial influence, and thus we hypothesize that an early-life exercise altered gut may contribute to the enduring neural adaptations produced by exercise. Using an oral antibiotic regimen to deplete gut bacteria, we therefore tested whether the presence of microbiota and associated metabolites is necessary to produce stress resistance. Juvenile rats ran or remained sedentary for three weeks. During this time, runner and sedentary rats were simultaneously given antibiotics. Following three weeks of exercise and antibiotics, wheels were locked with metal stakes, and 15 days later, all rats were exposed to inescapable stress (IS) and subsequent testing for IS-induced depressive-like behavior. Antibiotics administered along with exercise blocked the ability of exercise to protect against IS-induced depressive-like behavior. Next, we began to tease apart whether gut bacteria are necessary for the development versus the expression of these stress protective effects, and furthermore, whether the gut bacteria are important for both the immediate and longer lasting stress protective-effects of early life exercise. In order to determine whether gut bacteria are necessary for the development of the immediate stress-protective effects of exercise, we allowed juvenile rats to run for six weeks, and simultaneously administered oral antibiotics during just the first three weeks of exercise. Rats were then allowed to exercise for the latter three weeks sans antibiotics, so that their gut bacteria could reconstitute by the time of

testing. Following six weeks of exercise, all rats underwent IS and subsequent testing for depressive-like behavior. Antibiotics during the first three weeks of exercise did not block the development of early life exercise-induced stress resistance, at least when rats were tested immediately. Further research is necessary to determine whether antibiotics during the first three weeks of exercise can block the persistent stress protective effects. Furthermore, future research should also determine whether the presence of gut bacteria at the time of testing is necessary for their expression.

ii. Introduction

A constantly evolving literature demonstrates that gut microorganisms are critical for health (Eckburg et al., 2005). Microbial depletion models, such as rodents raised in sterile environments (germ free rodents; GF rodents) or given antibiotics, demonstrate that this ecosystem is critical for certain immune (Hooper et al., 2003; Hrncir et al., 2008; Ohara et al., 2000; Sudo et al., 1997; Tlaskalova-Hogenova et al., 2004; Berg & Garlington, 1979; Reikvam et al., 2011) and metabolic (Ajslev et al., 2011; Cox et al., 2014; Murphy et al., 2014; Conly et al., 1994; Hill, 1997; Sommer & Backhed, 2013) functions, and can even impact certain aspects of brain function and behavior (G. Clarke et al., 2013; Diaz Heijtz et al., 2011; Stilling et al., 2015; Sudo et al., 2004; Naseribafrouei et al., 2014; Desbonnet et al., 2014). Furthermore, particular species have the ability to enhance mood, behavior and brain function in both rodents (Savignac et al., 2015; Bravo et al., 2011) and humans (Steenbergen et al., 2015; Tillisch et al., 2013; Rao et al., 2009). Moreover, some studies suggest that gut microbe-brain interactions during sensitive developmental windows in early life can be more effective at altering mood and behavior (Stilling et al., 2015; Sudo et al., 2004; Desbonnet et al., 2015; Clarke et al., 2013). Notably, amplified HPA responses following stress in GF mice can be partially attenuated with bacteria from conventionally raised mice, but only if administered within six weeks of life (Sudo et al., 2004), while a long-term oral antibiotic regimen, administered immediately post weaning during the juvenile period, produced extensive depletion of the gut microbiota as well as abnormalities in neurochemistry and behavior in adulthood (Desbonnet et al., 2015). This literature demonstrates a link between gut microbes and the brain, and implicates early development as a potential window during which microbes exert the greatest influence.

Mechanisms underlying this complex, bidirectional, potentially age-dependent relationship remain elusive. Microbially-derived metabolites such short chain fatty acids (SCFAs) pose a promising potential signal between the gut and the central nervous system. Recent studies demonstrate that prebiotics, non-digestible complex carbohydrates that are fermented into SCFAs within the colon (Macfarlane et al., 2006; Vitali et al., 2012), can attenuate stress-induced anxiety (Tarr et al., 2015; Mika et al., 2016). The SCFA butyrate stands out due to the large body of work demonstrating its ability to benefit immune function (Furusawa et al., 2013), metabolism (Gao et al., 2009; Ridaura et al., 2013; Korecka et al., 2013) and importantly, brain plasticity, memory, mood and behavior (extensively reviewed in Stilling et al., 2016; Schroeder et al., 2007; Intlekofer et al., 2013). Although the majority of this work utilizes supraphysiological doses of butyrate administered through i.p. injection or ingestion, recent work has demonstrated the potent benefits of microbially-derived butyrate. For instance, microbial butyrate is critical for serotonin biosynthesis within intestinal enterochromaffin cells (Yano et al., 2015), which are responsible for 90% of the hosts' serotonin production. Another recent study demonstrated that increased blood brain barrier (BBB) permeability within GF mice can be completely restored by introducing either butyrate-producing bacteria or butyrate alone (Braniste et al., 2014). Collectively, this work highlights butyrate as a potential gateway between gut microbiota and the CNS.

Investigating the mechanisms by which exercise can protect the brain against the detrimental behavioral effects of inescapable stress (IS) has been a major focus for our lab. Exercise can increase beneficial bacteria in the gut (S. F. Clarke et al., 2014; Evans et al., 2014; Kang et al., 2014; Matsumoto et al., 2008; Petriz et al., 2014; Queipo-Ortuno et al., 2013; Santacruz et al., 2009), including the probiotic *Lactobacillus* and *Bifidobacteria* spp. (Queipo-

Ortuno et al., 2013) and butyrate-producing bacteria (Matsumoto et al., 2008). Furthermore, we have shown that exercise initiated in early life has a more profound impact on the gut than exercise initiated in adulthood (Mika & Fleshner, 2016; Mika et al., 2015), and can increase probiotic and butyrate-producing bacteria (Mika & Fleshner, 2016) as well as butyrate itself (Mika & Fleshner, 2016b). We have also demonstrated that exercise initiated during early, critical periods can produce more persistent and robust neurobiological and behavioral effects than exercise initiated in adulthood. Specifically, when exercise is initiated during the juvenile period, transient periods of exercise produce longer lasting protection against IS-induced anxiety and depressive-like behavior. When exercise is initiated in adulthood, however, rats require longer periods of exercise in order to develop resistance to IS-induced behaviors and must continue to exercise in order to maintain such benefits (Mika et al., 2016, in preparation). These robust behavioral and neurobiological effects could partially be due to the unique changes in gut bacteria produced by exercise in early life.

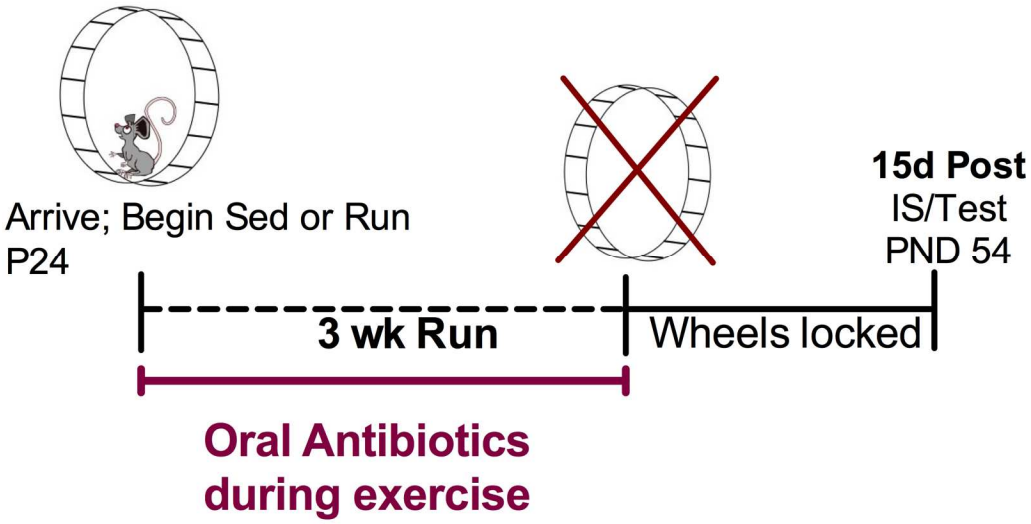
Manipulations that increase adaptive bacterial species within the gut in early life are sufficient to produce protection against the behavioral consequences of IS; we've recently shown that four weeks of prebiotic diets, fed during the juvenile period, increase *Lactobacillus* spp. and attenuate IS-induced anxiety and depressive like behavior. In order to determine if similar adaptations in gut microbiota are necessary for exercise-induced stress resistance, we conducted a series of experiments where we allowed juvenile, male F344 rats to exercise while simultaneously administering a broad spectrum oral antibiotic regimen, and subsequently, examining IS-induced depressive-like behavior.

iii. Material and methods

a. Experimental design

Experiment 1. We first tested whether the presence of microbiota and microbial metabolites is necessary to produce long lasting stress resistance. Juvenile rats ran or remained sedentary for three weeks, beginning at PND 24. During this time, runner and sedentary rats were simultaneously given three weeks of antibiotics. We chose three weeks because this exercise time course is sufficient to produce long-lasting protection against IS-induced depressive-like behavior (Mika et al., in preparation). Furthermore, we've also demonstrated that increases in probiotic bacteria (Mika et al., 2015; Mika et al., 2016a) and butyrate (Mika et al., 2016b) following juvenile onset exercise occur within three weeks of exercise. Following three weeks of exercise and antibiotics, wheels were locked with metal stakes for 15 days. Subsequently, all rats were exposed to inescapable stress (IS) and subsequently tested for IS-induced depressive-like behavior.

Figure 1

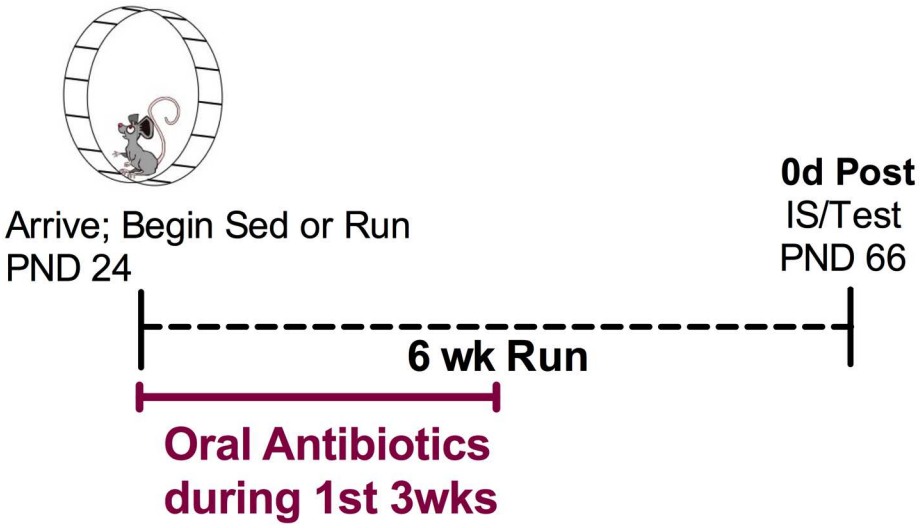


Experimental timeline. To examine whether the presence of microbiota and microbial metabolites is necessary to produce long lasting stress resistance, juvenile rats ran or remained sedentary for three weeks, beginning at PND 24. During this time, runner and sedentary rats were simultaneously given three weeks of antibiotics. Following three weeks of exercise and antibiotics, wheels were locked with metal stakes for 15 days. Then, all rats were exposed to IS and subsequently tested for IS-induced depressive-like behavior.

Experiment 2. In the first experiment, we weren't able to assess shuttle box escape behaviors immediately following exercise due to the young age of the rats at the time exercise was completed; we know from previous experiments (data not shown) that IS may not produce significant deficits in shuttle box escape instrumental learning at this age. Thus, we were unable to discern whether the presence of the gut bacteria is necessary for the stress protective effects that are present immediately following exercise, or for the longer lasting effects that are unique to juvenile-onset exercise. Furthermore, using this design, it is also unclear whether gut bacteria are necessary for the development of the stress protective effects versus their expression.

In order to begin to tease apart whether gut bacteria are necessary for the development versus the expression of the stress protective effects of early life exercise, and whether the presence of the gut bacteria during early life running is necessary for the immediate versus the longer lasting effects, we allowed juvenile rats to run for six weeks and we simultaneously administered oral antibiotics during the first three weeks of exercise only. This design will allow us to test rats immediately after exercise cessation; after six weeks of exercise, juveniles will have reached early adulthood, an age during which IS reliably produces depressive-like behaviors. Following three weeks of exercise and antibiotics, we then allowed rats to exercise for three weeks more weeks without antibiotics, so that depleted gut bacteria could reconstitute by the time of testing. In order to ensure proper reconstitution, we introduced feces from non-antibiotic rats (with the same exercise status) into the cages of the antibiotic rats immediately following completion of the antibiotic regimen. Following six weeks of exercise, all rats underwent IS, and were subsequently tested for depressive-like behavior.

Figure 2



Experimental time. In order to determine whether gut bacteria are necessary for the development of the immediate stress protective effects of early life exercise, we allowed juvenile rats to run for six weeks and we simultaneously administered oral antibiotics during the first three weeks of exercise only. Following three weeks of exercise and antibiotics, we then allowed rats to exercise for three weeks more weeks without antibiotics, so that depleted gut bacteria could reconstitute by the time of testing. In order to ensure proper reconstitution, we introduced feces from non-antibiotic rats (physical activity status matched) into the cages of the antibiotic rats immediately following completion of the antibiotic regimen. Following six weeks of exercise, all rats underwent IS, and were subsequently tested for depressive-like behavior.

b. Rats and wheel running

Juvenile (PND 24) male Fischer 344 rats (Envigo; Indianapolis, IN) were pair-housed (12:12 h light/ dark cycle in humidity and temperature (22°C) controlled housing) in either sedentary (standard Nalgene Plexiglas cages; 45cm × 25.2cm × 14.7cm) or running conditions (standard cage equipped with a running wheel (Mini Mitter Instruments, Bend, OR, USA). Wheel revolutions were digitally recorded via Vital View software (Mini Mitter, Bend, OR, USA), and average nightly running distance per week, estimated per rat, was calculated by multiplying the number of wheel revolutions by the circumference of the running wheel (1.081 m). All rats were allowed ad libitum access to food and water for the duration of each study. Food was measured weekly, and all rats were weighed at this time. Fecal samples were collected from rats once per week, at the time of weighing. Liquid consumption was measured on a daily basis, and antibiotic cocktail was freshly prepared every 24 hours. The University of Colorado Animal Care and Use Committee approved all protocols for these experiments, and care was taken to ensure minimal discomfort during all procedures.

c. Antibiotic regimen

This broad-spectrum antibiotic regimen (consisting of 4.0 mg/ml streptomycin and 2.0 mg/ml penicillin) has been previously shown to significantly reduce gut bacteria (Ammor et al., 2007; Maslanik et al., 2012). The antibiotic cocktail was dissolved in drinking water and given ad libitum. Based on our previous work (Maslanik et al., 2012), rats will readily drink this antibiotic cocktail and tolerate the short-term microflora depletion well (i.e., no measurable body weight loss). The use of oral antibiotic regimens offers a targeted, clinically applicable model to study the effects of microbial species upon host physiology.

d. Inescapable stress

Rats received 100, 1 mA inescapable tail shocks over a 2-hour period. Tail shocks were administered via local electrodes, at variable intervals (VI of 60 s); rats were restrained in Broome-style Plexiglas tubes (23.4 cm in length and 7.0 cm in diameter) with tails exposed for electrode attachment. Shock intensity increased from 1.0 to 1.5mA after the first 50 shocks to account for stress-induced analgesia. Immediately following IS termination, rats were returned to their home cage. This procedure occurred during the inactive (light) cycle from 0800 to 1000.

e. Shuttle box escape testing

24 hours following IS, rats were assessed for depressive-like behavior with the shuttle box escape behavioral tasks. This task measures deficits in instrumental learning that have been previously associated with clinical depression. In accordance to previous protocols (Mika et al., 2016), shuttle box escape deficits were assessed during the inactive cycle from 0800 to 1200. Briefly, rats were placed in shuttle boxes (50.8cm × 25.4cm × 30.48cm, Coulbourn Instruments, Whitehall, PA) and allowed a 10-minute exploratory period. Rats then received 2 fixed ratio 1 (FR-1) foot shocks (0.1 mA, 60 s ITI), where shock was continually administered until the rat crossed from one side of the shuttle box to the other. Subsequently, rats received 25 fixed ratio 2 (FR-2) foot shocks (0.6 mA, 60 s ITI). This time, shock was continuously administered until the rat crossed from one side of the shuttle box to the other twice. If a rat failed to cross twice within 30 seconds, a latency score of 30 was given, and the shock was terminated. Latencies to cross were recorded by a blind experimenter during FR-1 and FR-2 administration.

f. Fecal butyrate measurement

Fecal samples were collected following 3 weeks of exercise and antibiotic administration were sent to the Metabolomics Core at the University of Michigan for analysis of butyrate. Samples were processed using reversed-phase liquid chromatography-mass spectrometry,

in accordance to previous protocols (Chassaing et al., 2015).

g. Selective Bacterial culture

Lactobacillus-specific culture media (modified-rhamnose-2,3,5-triphenyltetrazolium (TTC) chloride-LBS-vancomycin agar; M-RTLV-agar) was used to selectively measure *L. rhamnosus* and *L. casei*, *L. paracasei*, *L. plantarum*, *L. fermentum*, *L. reuteri*, *L. sakei*. Briefly, M-RTLV agar allows for selective growth of vancomycin resistant *Lactobacillus* spp. and allows for visual discrimination of *L. rhamnosus* colony forming units (CFU) from other Lactobacillus spp. due to the fermentative capabilities of *L. rhamnosus*. M-RTLV agar was prepared by combining L-rhamnose (0.4 g/mL), TTC (30.0 mg/mL), vancomycin (10.0 mg/mL), and metronidazole (10.0 mg/mL) with nutrient agar, as specified in previously published protocols (Mika et al., 2016).

Total aerobic and total anaerobic bacteria were cultured using nutrient agar (Difco Nutrient Agar, BD; 23g/L).

Briefly, fecal samples were homogenized (0.2g of each sample in 2.0 mL phosphate buffered saline), and subsequently diluted in phosphate buffered saline (1:5000), then plated. Plated samples were incubated at 37° C; *Lactobacillus* spp. and anaerobic bacteria were incubated in anaerobic conditions created by a BD GasPak EZ Anaerobe Container System. Colony forming units (cfu) were counted and dilution corrected averages were then calculated and analyzed.

h. Statistical Analyses

Body weight was analyzed using 2 (antibiotic) x 2 (exercise) x time repeated measures ANOVAs. Running distances were summed nightly during the active cycle, and further summed to produce total distance per week. These values were divided by two to estimate distance per

rat, and analyzed using a 2 (antibiotic) x 6 (week 1- 6) or 3 (week 1-3) repeated measures ANOVA. Mean shuttle box escape scores were analyzed using a 2 (stress) x 2 (antibiotic) x 2 (exercise) ANOVA. Butyrate and bacteria cfu were analyzed using a 2 (antibiotic) x 2 (exercise) ANOVA.

iv. Results

a. Body weight, running, food & liquid consumption

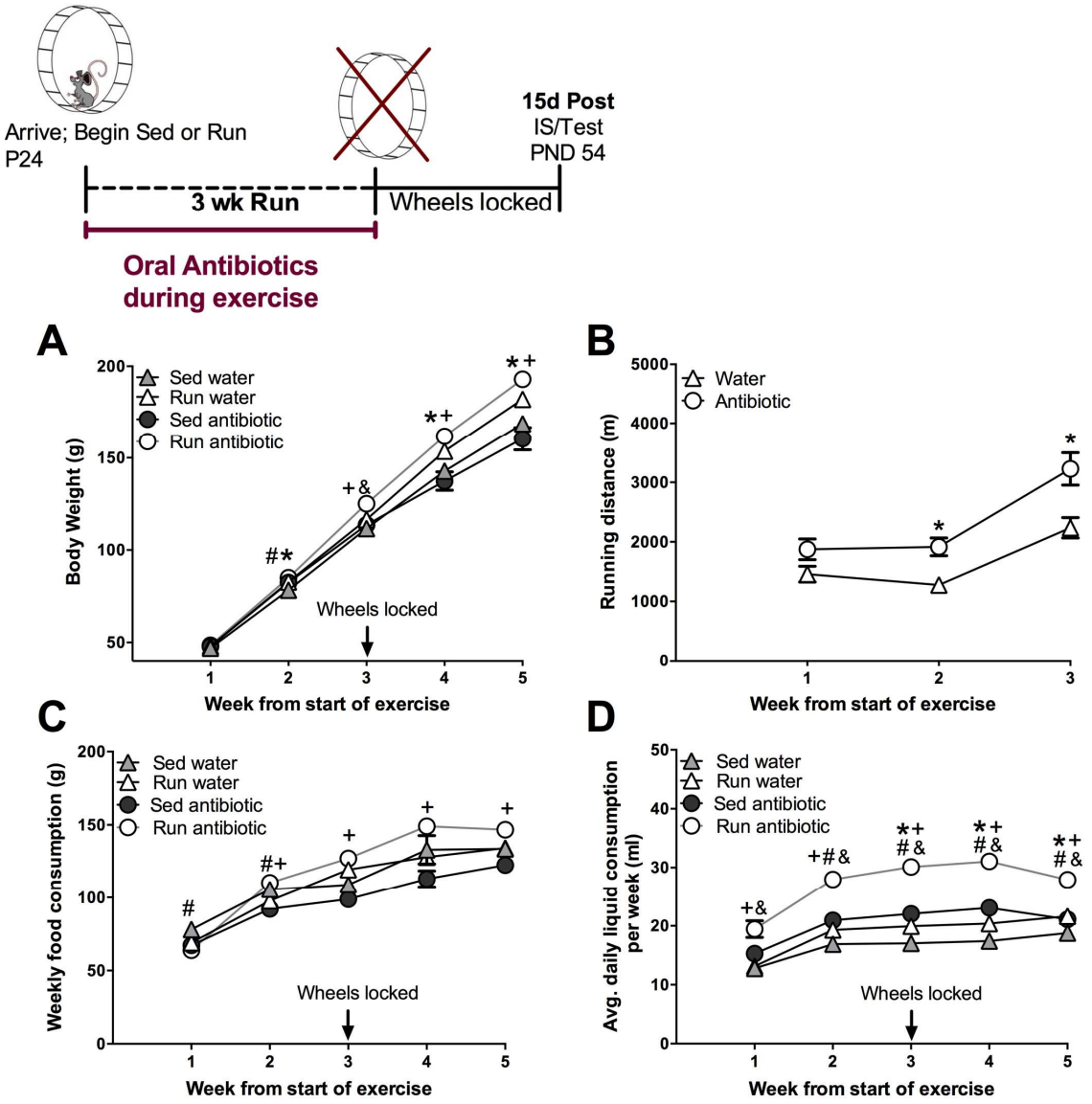
Body weight. Body weight increased across time ($F(4,176)=2047.766$; $p<0.0001$). Overall, antibiotics did not impact body weight ($F(1,176)=1.804$; $p=0.1861$), while exercise increased it ($F(1,176)=36.188$; $p<0.0001$). A time x antibiotic x exercise interaction ($F(4,176)=3.927$; $p=0.0044$) and subsequent post hocs further revealed that exercised rats were significantly heavier than other groups throughout the study, even after wheels were locked. Furthermore, exercised rats receiving antibiotics demonstrated the highest body weight compared with all other groups at certain time points. See graph for detailed post hoc comparisons.

Exercise. Running distance increased across time ($F(2,44)=53.042$; $p<0.0001$), and antibiotics increased running distance overall ($F(1,44)=11.103$; $p=0.0030$). Post hocs further revealed that antibiotics significantly increased running distance during the second and third week of exercise.

Food consumption. Food consumption increased across time ($F(4,176)=316.409$; $p<0.0001$), and exercise increased food consumption overall ($F(1,176)=15.155$; $p=0.0003$). A time x antibiotic x exercise interaction ($F(4,176)=4.818$; $p=0.0010$) further revealed that exercised rats receiving antibiotics demonstrated the highest food consumption during certain time points.

Liquid: Liquid consumption increased across time ($F(4,176)=127.982$; $p<0.0001$) and both antibiotics ($F(1,176)=236.514$; $p<0.0001$) and exercise ($F(1,176)=129.192$; $p<0.0001$) increased liquid consumption overall. Antibiotic x exercise ($F(1,176)=31.428$; $p<0.0001$), time x antibiotic ($F(4,176)=9.865$; $p<0.0001$) and time x exercise ($F(4,176)=5.382$; $p=0.0004$) interactions and subsequent post hocs revealed that exercising rats given antibiotics consumed significantly more liquid than the other group throughout the duration of the study.

Figure 3

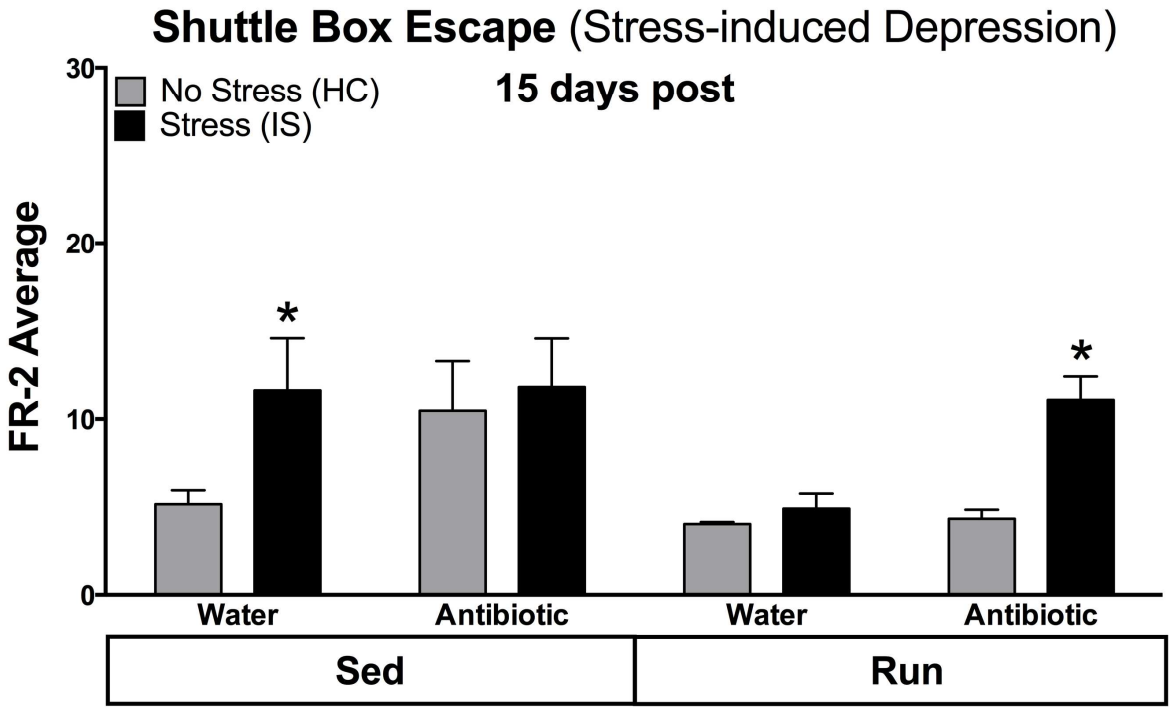


Body weight, running, food & liquid consumption. A) Body weight across the experiment. B) Running distance, measured as average total distance per week, estimated per rat, across three weeks of exercise. C) Weekly average food consumption, estimated per rat. D) Average daily liquid consumption per week, estimated per rat. Data are represented as mean ± SEM. N: 6-8/grp. *p<0.05; * indicates differences between sed and run water; + indicates difference between sed and run antibiotic; # indicates difference between water sed and antibiotic sed; & indicates difference between water run and antibiotic run.

b. Antibiotics during exercise blocked exercise-induced protection against IS-induced instrumental learning deficits

Overall, exercise ($F(1,37)=5.881$; $p=0.0203$) decreased escape latency, while antibiotics ($F(1,37)=5.282$; $p=0.0273$) and stress ($F(1,37)=6.578$; $p=0.0145$) increased it. Subsequent post hocs revealed that antibiotics increased escape latency in non-stressed sedentary rats. Juvenile runner rats that drank water throughout three weeks of exercise were successfully protected against IS-induced increases in escape latency. However, exercise did not protect against IS-induced increases in escape latency in the juvenile runner rats given antibiotics.

Figure 4



Antibiotics during exercise blocked exercise-induced protection against IS-induced instrumental learning deficits. A) average escape latency across the entire testing session. Data are represented as mean ± SEM. *$p < 0.05$. N: 6-8/grp.

c. Body weight, running, food consumption, liquid consumption

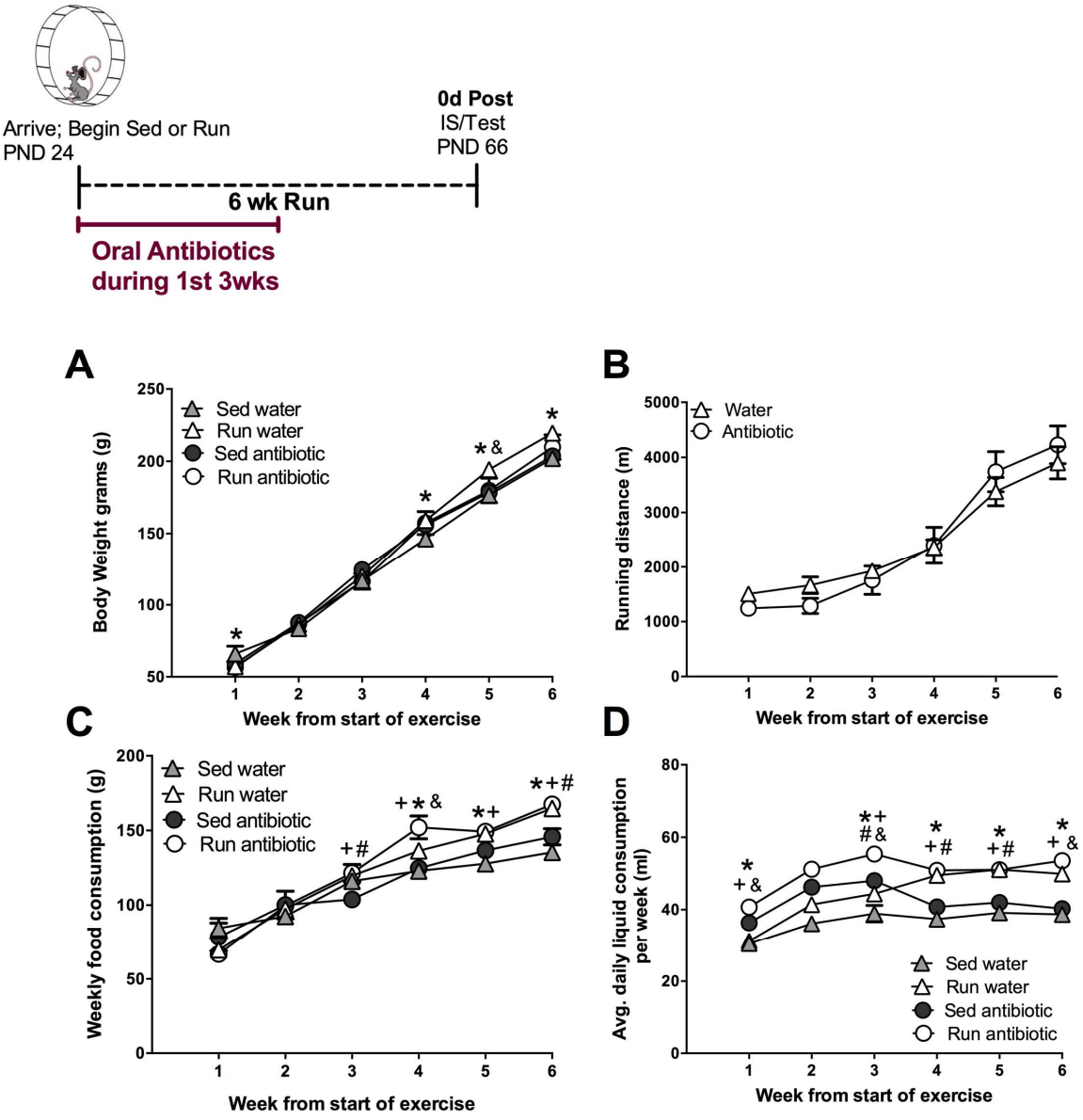
Body weight. Body weight increased over time ($F(5,215)=2775.880$; $p<0.0001$), and overall, antibiotics ($F(1,215)=0.080$; $p=0.7780$) and exercise ($F(1,215)=1.080$; $p=0.3046$) did not impact body weight. A time x exercise ($F(5,215)=10.421$; $p<0.0001$) as well as a time x antibiotic x exercise ($F(5,215)=3.444$; $p=0.0052$) revealed that exercised rats demonstrated the highest body weight compared with other groups at certain time points. See graph for detailed post hoc comparisons.

Exercise. Running distance increased over time ($F(5,110)=92.082$; $p<0.0001$), and antibiotic administration during the first three weeks of exercise did not impact running distance ($F(1,110)=0.005$; $p=0.9428$).

Food consumption. Food consumption increased over time ($F(5,220)=224.669$; $p<0.0001$), and antibiotics did not impact overall food consumption ($F(1,220)=0.984$; $p=0.3266$), while food consumption increased in exercising rats ($F(1,220)=15.256$; $p=0.0003$). A time x antibiotic ($F(5,220)=2.251$; $p=0.0504$) as well as a time x exercise ($F(5,220)=12.884$; $p<0.0001$) interaction further revealed that at certain time points, exercised rats, receiving either antibiotics or water, demonstrated the highest food consumption.

Liquid consumption. Liquid consumption increased over time ($F(5,220)=124.346$; $p<0.0001$), and overall, both antibiotic administration ($F(1,220)=55.821$; $p<0.0001$) and exercise ($F(1,220)=111.219$; $p<0.0001$) increased liquid consumption, and a time x antibiotic x exercise ($F(5,220)=2.553$; $p=0.0286$) interaction and subsequent post hocs further revealed that exercising rats, given either water or antibiotics, consumed significantly more liquid than the other groups at certain time points.

Figure 5

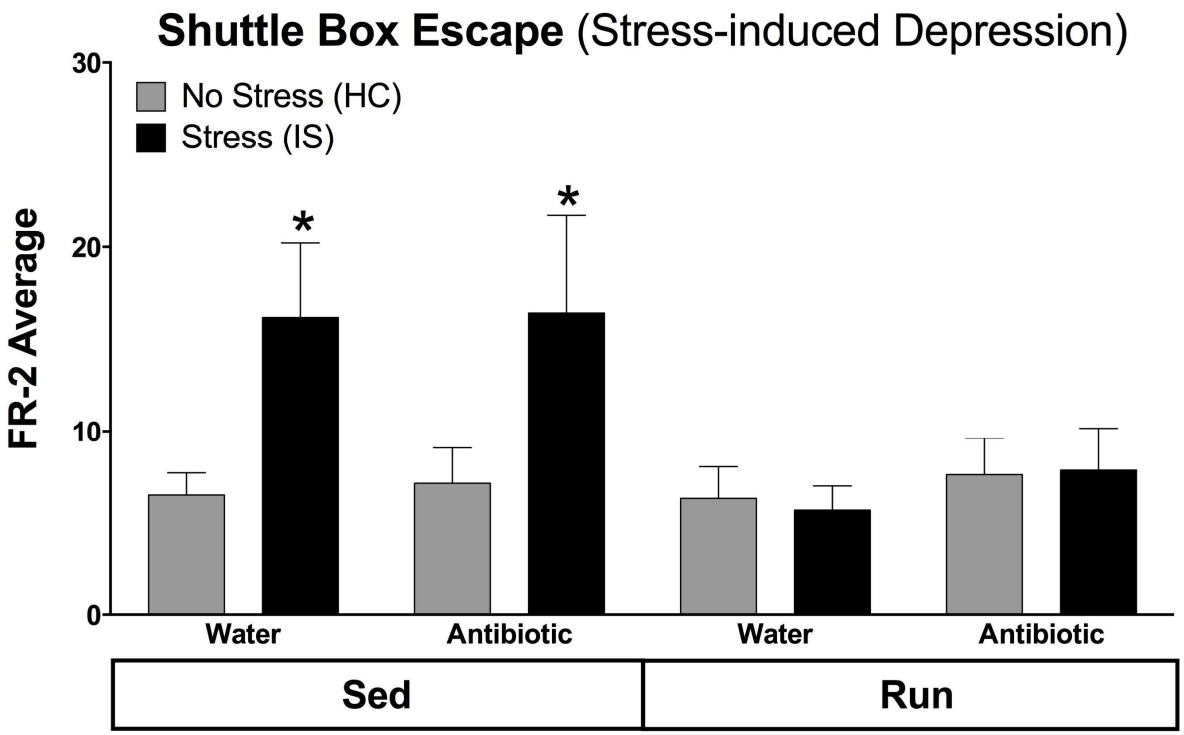


Body weight, running, food & liquid consumption. A) Body weight across the experiment. B) Running distance, measured as average total distance per week, estimated per rat, across six weeks of exercise. C) Weekly average food consumption, estimated per rat. D) Average daily liquid consumption per week, estimated per rat. Data are represented as mean ± SEM. N: 6-8/grp.*$p<0.05$; * indicates differences between sed and run water; + indicates difference between sed and run antibiotic; # indicates difference between water sed and antibiotic sed; & indicates difference between water run and antibiotic run.

d. Antibiotics during first three weeks of exercise did not block Antibiotics during exercise blocked exercise-induced protection against IS-induced instrumental learning deficits

Overall, exercise ($F(1,37)=7.325$; $p=0.0102$) decreased escape latency in the shuttle box escape task, while antibiotics had no effect ($F(1,37)=0.112$; $p=0.7401$). A run x stress interaction ($F(1,37)=5.302$; $p=0.0270$) and subsequent post hocs further revealed that exercise, regardless of antibiotics, protected rats against the IS-induced increases in escape latency observed in sedentary rats.

Figure 6

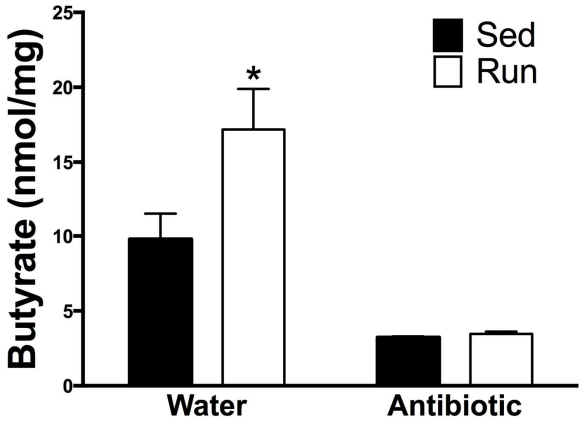


Antibiotics during first three weeks of exercise did not block exercise-induced protection against IS-induced instrumental learning deficits. A) average escape latency across the entire testing session. Data are represented as mean ± SEM. *$p < 0.05$. N: 6-8/grp.

e. *Exercise increases, while antibiotics reduce, probiotic bacteria and butyrate*

Fecal butyrate. Overall, exercise increased butyrate ($F(1,17)=6.879$; $p=0.0177$) while antibiotics significantly reduced it ($F(1,17)=48.795$; $p<0.0001$). An exercise by antibiotic interaction was detected ($F(1,17)=6.085$; $p<0.024$) and post hocs further revealed that butyrate was significantly increased following exercise in the rats that drank water, compared with sedentary water rats.

Figure 7

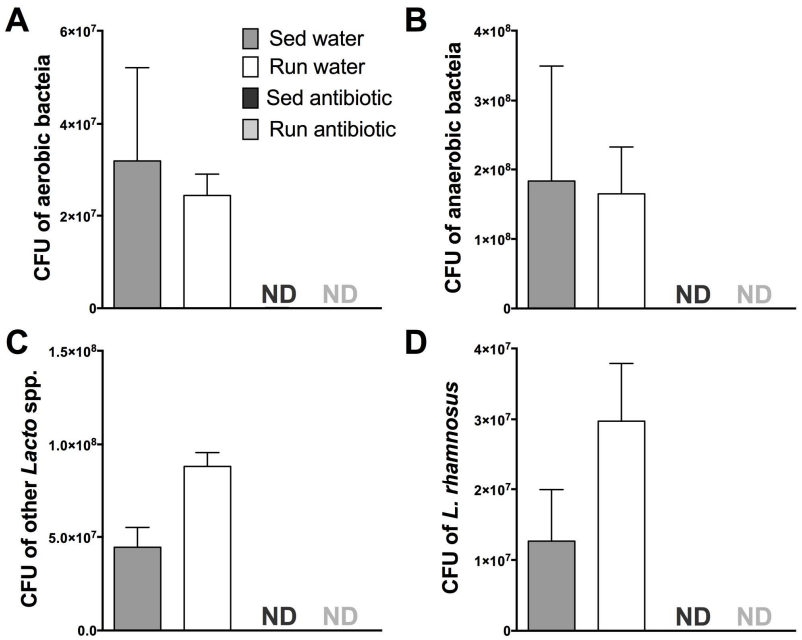


Exercise increases, while antibiotics reduce, butyrate. Fecal butyrate levels (nmol/mg), following three weeks of exercise and antibiotics, taken from a subset of rats. Data are represented as mean ± SEM. *$p < 0.05$. N: 6-8/grp.

Aerobic and anaerobic bacteria and *Lactobacillus* spp. Exercise had no effect on aerobic ($F(1,8)=0.395$; $p=0.5474$) or anaerobic bacteria ($F(1,8)=0.031$; $p=0.8642$), and following the antibiotic regimen, no colony forming units were detected.

Exercise increased other *Lactobacillus* spp. ($F(1,6)=41.107$; $p=0.0007$) as well as *L. rhamnosus* ($F(1,7)=7.905$; $p=0.0261$). Similarly, no colony forming units were detected following the antibiotic regimen.

Figure 8



Exercise increases, while antibiotics reduce, probiotic bacteria. A) Aerobic and B) anaerobic bacteria and C)-D) *Lactobacillus* spp., from a subset of rats following 1 week of exercise and antibiotics. C) *L. casei*, *L. paracasei*, *L. plantarum*, *L. fermentum*, *L. reuteri*, *L. sakei*. are grouped together as "other lacto." Data are represented as mean ± SEM. *$p < 0.05$. N: 2-3/grp.

v. Discussion

In our first experiment, we demonstrate that the presence of the gut bacteria is important for the stress protective effects of early life exercise; antibiotics simultaneously administered throughout the entire three weeks of exercise prevents exercise-induced protection against depressive-like behavior 15 days later.

However, using this experimental approach, it is unclear whether the presence of the gut bacteria is important for the immediate stress protective effects of exercise versus the unique, longer lasting effects that are only present following juvenile-onset exercise. Prior data demonstrate that IS does not reliably produce deficits in shuttle box escape instrumental learning around the young age of PND 45, thus shuttle box escape behaviors immediately following exercise were not assessed.

We hypothesize that the immediate effects stress protective effects of exercise are mechanistically distinct from the longer lasting effects for several reasons. First, physically active rats, regardless of age, are protected against IS-induced anxiety and depressive-like behaviors immediately following exercise. In adults, these effects are transient, and require six weeks of exercise to develop. However, if exercise is initiated in early life, the stress protective effects last even following removal of wheel access, and shorter periods of exercise can produce these effects. Moreover, juvenile-onset runners also exhibit robust, distinctive changes in gene expression. The distinct behavioral and neurobiological profiles of young versus adult runners suggest that age-dependent mechanisms are at play. Furthermore, exercise initiated in the juvenile period is capable of producing pronounced shifts in gut microbial ecology, as evidenced by changes in bacterial phyla (Mika et al., 2015), as well as increases in certain probiotic bacterial species (Mika et al., 2015; Mika et al., 2016a). In contrast, exercise in adulthood did not

produce pronounced change, although it was, to a smaller extent, able to impact certain species. Given that exercise in adult runners is capable of producing immediate protection against exercise-induced stress resistance, yet is incapable of significantly altering gut ecology or altering probiotic or butyrate-producing gut bacteria, we hypothesize that gut microbes may not necessarily contribute to the stress protective effects of exercise in adult rats.

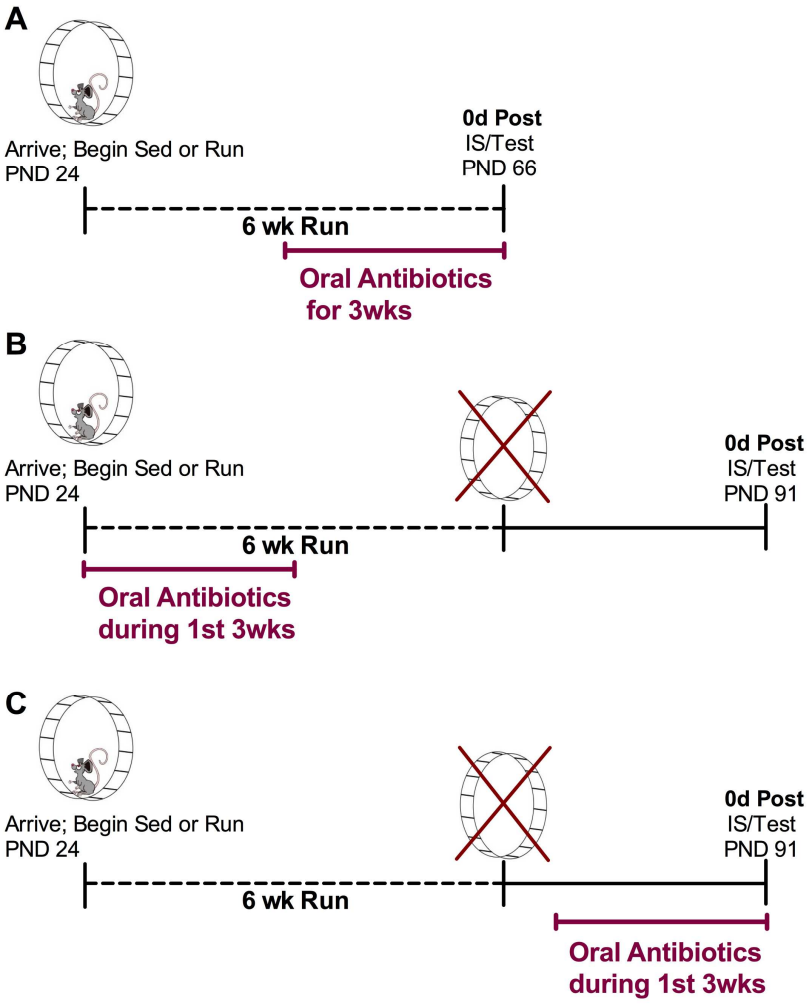
In addition, in the first experiment, it is unclear whether 15 days was sufficient to reconstitute gut flora by the time of testing. Previous work has shown that longer lasting antibiotic regimens, begun during the juvenile period, can produce persistent dysbiosis (Desbonnet et al., 2015). Thus, it is possible that gut ecology was still compromised by the time of testing, and we are unable to conclude whether or not gut bacteria is necessary or sufficient for the stress-protective effects of early life exercise.

We altered the design of our second experiment so that we could further understand these variables. We used six weeks of exercise instead of three, so that we would be able to selectively deplete gut bacteria earlier versus later to examine whether this ecosystem is necessary for the development versus the expression of the immediate stress-protective effects. Furthermore, we re-introduced fecal material from non-depleted, exercise-matched rats into the cages of depleted rats, immediately following the antibiotic regimen. Here, three weeks of antibiotics beginning during a six week running time course did not block the immediate stress-protective effects of exercise. It is possible that gut bacteria are not necessary for the development of these immediate effects. However, it is also possible that these protective effects were still able to develop in the latter three weeks of exercise.

There are three more depletion experiments that must be done in order to better understand these questions. First, using the same design as the second experiment, we must

deplete the gut microbiota during the latter three weeks of exercise in order to test whether gut bacteria are necessary for the expression of the immediate stress protective effects. It is also important to repeat these experiments in adult runners, to ensure that exercise-altered gut microbes are not contributing to the immediate stress protective effects produced by exercise in adulthood. Next, we must ask whether gut bacteria are important for the development or the expression of the longer lasting effects. To test this, rats will run for six weeks, after which wheels will be locked for 25 days; rats will undergo IS/ subsequent testing 25 days following cessation of exercise. Antibiotics will be administered either in the first three weeks of running, to test whether the gut microbiota are important for the development of these effects, or given following exercise cessation, to test if the gut is necessary for their expression.

Figure 9



Proposed experiments. A) we will deplete gut microbiota during the latter three weeks of exercise in order to test whether gut bacteria are necessary for the expression of the immediate stress protective effects of early life exercise (will be repeated in adults). To examine whether gut bacteria are important for the development or the expression of the longer lasting effects, rats will run for six weeks, after which wheels will be locked, and rats will undergo IS/ subsequent testing 25 days following cessation of exercise. Antibiotics will be administered either B) in the first three weeks of running, to test whether the gut microbiota are important for the development of these effect, or C) given following exercise cessation, to test if the gut is necessary for the expression of these long lasting effects.

Another major flaw with our approach is the use of a depletion model. There are many negative physiological effects associated with long term antibiotics use, any of which could produce a major confound. Indeed, in the first experiment, three weeks of antibiotics for the entire duration of exercise increased depressive-like behavior in non-stressed sedentary rats, and altered running distance, food and liquid consumption. Another way to examine whether a microbial ecosystem, altered by early life exercise, is important exercise-induced stress protection is by giving sedentary rats feces from physically active rats in early life. This would test whether an exercise-altered gut is sufficient to produce stress resistance in early life.

Though our experiments are not designed to test how microbes and associated microbial metabolites communicate with the CNS to produces stress resistance, there are many possible mechanisms by which this can occur, and these are not likely mutually exclusive. Of microbial metabolites, early life exercise-induced increases in butyrate highlight this SCFA as a promising contender. Butyrate acts as a histone deacetylase inhibitor, and a large literature demonstrates the use of butyrate as a tool to produce adaptive alterations in gene expression and behavior (reviewed extensively in Stilling et al., 2016). However, these studies utilize exogenous butyrate, and it is unclear whether endogenous butyrate, derived from microbial fermentation, is able to travel beyond the intestines and impact other aspects of host physiology. Others have pointed out that this is unlikely, given that prior studies have reported that a small amount of butyrate gets absorbed into the circulation (studies have detected anywhere from 5-7%; den Besten et al., 2013). However, a recent study demonstrated that blood brain barrier (BBB) permeability within GF mice was significantly increased, as evidenced by decreased expression of endothelial tight junction proteins claudin-5 and occludin (Braniste et al., 2014). When GF mice were colonized with certain butyrate-producing bacterial species, BBB integrity/tight junction protein expression

was restored. The authors further demonstrated that butyrate supplementation alone was sufficient to restore BBB integrity within GF mice, indicating that butyrate is indeed capable of interacting with host tissues other than EC cells, and is indeed capable of interacting with the CNS. Thus, it is not far-fetched to hypothesize that endogenous butyrate is also capable of modulating brain function.

The butyrate that gets absorbed the circulation is capable of influencing brain function directly, by passing through the blood brain barrier, or indirectly by producing adaptations in peripheral physiology that can subsequently influence brain function. The passage of butyrate into the brain can occur through passive diffusion, given that it is a hydrophobic molecule that can cross through lipid bilayers, or through transport proteins, of which there are several (Stilling et al., 2016). Using an in vitro model of the BBB, one study demonstrated that butyrate can readily diffuse across the BBB, but this attenuated when the transport protein cluster of differentiation 36 (CD36) is blocked (Mitchell et al., 2011).

Interestingly, butyrate may not have to travel to the CNS in order to produce stress resistance. Butyrate administration can increase the transcriptional co-activator peroxisome proliferator-activated receptor-γ coactivator α (PGC-1α). PGC-1α is also increased in skeletal muscle following exercise-induced muscle contractions (Baar et al., 2002), and subsequent PGC-1α interactions with the nuclear receptor family peroxisome proliferator-activated receptors (PPARs) has long been implicated as a primary mechanism by which exercise produces physiological adaptations in skeletal muscle and host metabolism. By increasing PGC-1α mRNA and protein with the muscle, butyrate itself can initiate pathways that produce some of the same metabolic adaptations as endurance exercise. For instance, one study showed that butyrate-induced increases in PGC-1α increased mitochondrial biogenesis within skeletal muscle

(Gao et al., 2009). Most intriguingly, butyrate-induced increases in PGC-1α in skeletal muscle can also influence brain function. PGC-1α activation of the PGC-1α-PPARα/δ pathway can increase expression of kynurenine aminotransferases within skeletal muscle. These enzymes are responsible for converting kynurenine into kynurenic acid, thus decreasing the amount of peripheral kynurenine, a metabolite that readily crosses the BBB, and increasing kynurenic acid, a metabolite that cannot cross the BBB. Chronic stress-induced increases in kynurenic acid are responsible for producing depressive-like behaviors in a rodent model, and transgenic mice overexpressing skeletal muscle specific PGC-1α had decreased levels of peripheral kynurenine, increased kynurenic acid and kynurenine aminotransferases, and were protected against chronic stress-induced depressive-like behaviors (Agudelo et al., 2014). Given that butyrate can potently upregulate PGC-1α mRNA and protein within skeletal muscle, and can also bind to and activate PPAR (Alex et al., 2013), it is capable of mimicking the adaptive effects of exercise on host physiology.

Our lab has preliminary evidence to support that a different protein end product of the PGC-1α signaling pathway may be involved in mediating the exercise-induced resistance against IS-induced LH behaviors. Irisin is a novel PGC-1α-dependent signaling molecule released by skeletal muscle following exercise (Bostrom et al., 2012). A prior unpublished study from our lab demonstrated that systemic administration of irisin for 6 weeks through an osmotic mini-pump prevents IS-induced LH behaviors. We are not the only ones to observe neurobiological adaptations following peripheral irisin induction; indeed, another group demonstrated that adenoviral delivery of irisin may be mechanistically involved in producing exercise-induced increases in hippocampal BDNF (Wrann et al., 2013). In our model, the mechanisms by which irisin interacts with the brain to produce stress resistance are unknown, but could involve irisin-

induced increases in core body temperature, as elevating body temperature and activating the DRN through thermo-sensitive spinal nerves can have anxiolytic and antidepressant effects (Lowry et al., 2009). Importantly, given that microbially-derived butyrate can increase PGC-1α-dependent signaling pathways, it can potentially protect against IS-induced LH through the peripheral induction of the myokine irisin.

Other than butyrate, there are many other mechanisms by which the gut microbiota can interact with host brain function. Lactobacillus spp. and butyrate can alter brain function by promoting intestinal barrier integrity (Brahe et al., 2013; Yu et al., 2015). Bacterial translocation from intestines can activate immune cells and induce the release of pro-inflammatory cytokines, such as IL-6, IL-1β, TNFα, which can cross the BBB or communicate with the CNS through receptors on various afferent nerves (Dantzer et al., 2000). Increased bacterial translocation and subsequent immune activation has been previously associated with depressive symptoms (Dantzer et al., 2008; Maes et al., 2012). Gut microbes can also produce neurotransmitters and neurotransmitter-like substances, or alter metabolic pathways involved in neurotransmitter synthesis. A recent study demonstrated that Clostridium sporogenes ATCC 1579 possesses enzymes that can decarboxylate tryptophan into the neurotransmitter tryptamine (Williams et al., 2014; For a review of gut microbiota-derived neurotransmitters, see Sampson & Mazmanian, 2015). Although this body of work clearly demonstrates that certain gut microbes possess the enzymatic capabilities to produce or modulate the production of neurotransmitters, it is still unclear if these neurotransmitters and neurotransmitter-like substances can directly significantly affect CNS function. Though these complex interactions are not fully understood, these recent studies demonstrate that certain microorganisms produce enzymes and signaling molecules that can potentially interact with the brain through diverse pathways.

Several studies support the role of the vagus nerve as a key communication relay between the gut microbiota and the brain. For example, sub-diaphragmatic vagotomy dampens the impact of *Lactobacillus* spp. on the CNS (Bravo et al., 2011). Gut microorganisms can activate vagal afferents of the enteric nervous system through multiple mechanisms; various metabolites and signaling molecules that are produced by bacteria, including SCFAs via Gi- coupled receptor 41 (Nohr et al., 2015), as well as neurotransmitters and cytokines from immune cells, can activate adjacent receptors on vagal afferents. In the rat, the majority of vagal afferents originate in the cecum (which is also where the majority of microbial fermentation/ SCFA production occurs). Specifically, the celiac branches of the vagus originating from the cecum meet at the anterior vagal trunk, which bifurcates into the left vagus, and ascending vagal fibers on their way to the brain synapse onto the nodose ganglion (also known as the inferior ganglion of the vagus), which projects to the medial and commissural nuclei of the nucleus of the solitary tract (Altschuler et al., 1991). Subsequently, the solitary tract nucleus (NTS) receives microbiota-derived vagal signals and further propagates these signals to various projection regions, including the locus coeruleus (LC). NTS to LC projections are especially notable, since the LC shares direct connections with the dorsal raphe nucleus (DRN), a region of the brain important for mediating IS-induced depressive-like behavior. Indeed, vagus nerve stimulation increases the firing rate of both norepinephrine and serotonin neurons (Manta et al., 2009), importantly demonstrating that it may be possible for the gut microbiota to directly communicate with serotonergic circuits.

The current data presented in this report provide a starting point for future research. To further elucidate the biological mechanisms underlying the long lasting stress resistance produced by early life exercise, this work should aim to complete the experimental questions

posed in this discussion to determine what aspect of stress resistance gut bacteria are important for.

vi. References

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