Exercise during the Juvenile Period Produces Long Lasting Stress Protection Potentially Subserved by Epigenetic Alterations to the Serotonergic System

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Abstract

Exercise is capable of producing a myriad of beneficial alterations in brain chemistry and function which can help prevent stress-related psychiatric disorders, such as anxiety and depression. Recent work from our lab demonstrates that 6 weeks of exercise initiated in early life produces *lasting* protection against stress-induced anxiety and depressive like behaviors, whereas, in adults, this protection is transient and dissipates upon cessation of exercise. Thus, when exercise begins at a young age, the neurobiological improvements remain intact even after exercise has ceased for a period of time. The mechanism underlying this long lasting protection is currently unknown. Furthermore, it is unclear which particular developmental stage is sensitive to the unique, lasting effects of exercise. The purpose of this study was to investigate the underlying mechanisms by which this long lasting stress protection occurs, as well as the developmental stage vital for producing this protection. We investigated whether early life exercise produced lasting alterations in serotonin (5-HT) 1a autoreceptor (5-HT_{1a}R) mRNA levels; increases in mRNA for this receptor within the serotonergic dorsal raphe nucleus (DRN) are thought to be one mechanism by which exercise produces protection against the detrimental behavioral effects of stress. Juvenile, postnatal day 24 (PND 24), F344 rats ran for 6 weeks and mRNA expression levels of 5-HT_{1a}R were measured immediately and 25 days after exercise cessation. Exercise produced persistent increases in 5-HT_{1a}R mRNA levels within the DRN. We also investigated whether a shorter duration of exercise (3 weeks) restricted to the juvenile period could produce this unique lasting stress protection. Indeed, exercise restricted to the juvenile period

produces long lasting protection against the behavioral consequences of stressor exposure. Further, exercise during this period increases levels of butyrate, an endogenous histone deacetylase (HDAC) inhibitor. These data suggest that lasting alterations in gene expression may underlie these behavioral effects, and that the juvenile brain may harbor exceptional traits that allow for lasting plasticity. We conjecture that exercise-induced epigenetic modifications, specifically histone acetylation, during this developmentally sensitive time are involved in this long lasting increase in 5-HT_{1a}R mRNA. These results can inform treatments as well as prevention strategies for stress-related mental health disorders.

Introduction

Habitual, voluntary physical exercise produces an abundance of benefits, such as positive adaptations in both brain function and behavior. These adaptations include protecting the organism against stress-related psychiatric disorders such as anxiety and depression. Indeed, human and animal literature has shown that exercise can treat, as well as prevent, these stress related psychiatric disorders (Carek, Laibstain, & Carek, 2011; Greenwood, Foley, Burhans, Maier, & Fleshner, 2005; Greenwood et al., 2011; Krogh et al., 2014; Schmidt, 2010) Our lab has repeatedly shown that 6, but not 3, weeks of exercise in young adult rats (Greenwood, Foley, Burhans, et al., 2005) is sufficient to produce protection against the anxiety and depressive-like behaviors that are produced by uncontrollable, inescapable tail shock stress. In contrast, in sedentary rats, this uncontrollable, inescapable stressor (IS) produces anxiety and depressive-like behaviors that mirror human symptoms of stress-related psychiatric disorders, such as anxiety and depression. Specifically, IS in rodents produces both exaggerated fear responses to mild or benign stimuli and as well as deficits in instrumental learning tasks (Jackson, Alexander, & Maier, 1980; Maier, 1990). These stress-induced behaviors are also known as learned helplessness (LH) behaviors (Greenwood et al., 2003). Similarly, clinical studies show that anxious patients have exaggerated fear responses to mildly aversive stimuli (Jovanovic et al., 2009; Rauch et al., 2000) and that depressed patients commonly display deficits in instrumental learning (Tull, Barrett, McMillan, & Roemer, 2007). As we have demonstrated, altering the physical activity status of the rodent can

prevent these LH behaviors, and results from such studies can lend novel insights for the treatment and prevention of psychiatric disorders.

Importantly, we've recently demonstrated that this exercise-induced protection against LH behaviors is transient in adult rats (Mika, Bouchet, Spence, Greenwood, & Fleshner, 2013). After adult rats (PND 70) stop running, this protection disappears within 15 days of exercise cessation. Specifically, when adult rats run for 6 weeks then stop running for a period of 15 days or less, they will display LH behaviors typically seen if faced with IS. These data suggest that adults must continually exercise in order to remain protected from the behavioral consequences of stressor exposure.

Interestingly, when 6 weeks of exercise is initiated earlier in life during the juvenile period, starting at PND 24, the beneficial effects of exercise are persistent and stress protection remains long after exercise cessation and into adulthood. Specifically, when juvenile rats run for 6 weeks, then stop running, they remain just as protected against LH if faced with IS 15 days or 25 days after exercise cessation. These protective effects show no signs of attenuation at 25 days, suggesting the possibility that exercise produces protective effects in this age group that remain intact for much longer than investigated (Mika et al., 2013).

The mechanisms underlying this long lasting protection against LH behaviors in young runners are unknown. Previous work from our lab shows that exercise in young adulthood produces adaptive alterations in serotonergic (5-HT) circuits that control these stress-induced behavioral processes (Greenwood, Foley, Day, et al., 2005; Greenwood, Strong, et al., 2012). The dorsal raphe nucleus (DRN) is the main source of

5-HT in the brain, levels of which regulate emotional behaviors, such as anxiety and depression (Graeff, Guimaraes, De Andrade, & Deakin, 1996; Greenwood & Fleshner, 2011; Greenwood, Loughridge, Sadaoui, Christianson, & Fleshner, 2012; Soiza-Reilly & Commons, 2014). Exposure to IS leads to a hyperactivation of the 5-HT neurons within the DRN, resulting in an excessive release of 5-HT which desensitizes and downregulates 5-HT_{1a} inhibitory autoreceptors (5-HT_{1a}R) within the DRN (Albert, Le Francois, & Millar, 2011; Bravo, Dinan, & Cryan, 2014; Grahn et al., 1999; Rozeske et al., 2011; Sachs et al., 2013). Normally, 5-HT_{1a}R regulates 5-HT release, but when levels of these receptors are decreased, dysregulation and the subsequent high release of 5-HT during future stressful events leads to the development of LH behaviors (Albert et al., 2011; Graeff et al., 1996; Greenwood & Fleshner, 2011; Homberg, Molteni, Calabrese, & Riva, 2014; Maier & Watkins, 2005; Sachs et al., 2013). 6 weeks of exercise may prevent these LH behaviors by increasing 5-HT_{1a}R expression in the DRN. Exercised rats have higher levels of 5-HT_{1a}R and thus may be able to maintain proper constraint of 5-HT release during stressor exposure (Greenwood, Foley, Day, et al., 2005; Greenwood et al., 2003). Since altered 5-HT_{1a}R expression is implicated in exercise-induced stress protection in adults, it may also be important for the long lasting stress protection observed in juvenile rats.

As compared to humans, rodent development is highly accelerated (Romijn, Hofman, & Gramsbergen, 1991; Sengupta, 2013; Shaw et al., 2008). Therefore, 6 weeks of exercise initiated during early life, starting at PND 24 and specifically in male F344 rats, spans across multiple, discrete periods: the juvenile (analogous to childhood in humans; the period immediately post weaning to before puberty, approx. PND 24-45)

and adolescent (analogous to puberty and the years surrounding it in humans; beginning at slightly before puberty and lasting until early adulthood, approx. PND 42 to 50-60) developmental periods, as well as some early adulthood (approx. PND 60). Early life as a whole is a time of high plasticity resulting in massive synapse proliferation and subsequent pruning and reorganization of various neuronal circuits (Andersen, 2003; Andersen & Teicher, 2008; Hebbard, King, Malsbury, & Harley, 2003; Morrison, Rodgers, Morgan, & Bale, 2014; Sisk & Zehr, 2005; Spear, 2000). However, region specific maturation of various circuits occur during distinct stages of early life. For example, maturation of dopamine D2 receptors within the striatum occur during the juvenile period (Murrin & Zeng, 1986) whereas dopamine D1 and D2 receptors within the basal ganglia mature during the adolescent period (Rao, Molinoff, & Joyce, 1991). Interestingly, the juvenile period is also the time of 5-HT circuit maturation, particularly with 5-HT_{1a}R, where receptor expression is more sensitive to alterations and is still in the process of maturing to adult levels (Booij, Tremblay, Szyf, & Benkelfat, 2015; Sidor, Amath, MacQueen, & Foster, 2010). Due to this high level of plasticity in the juvenile period within regions shown to be important for exercise-induced stress protection in older animals, it could be that the juvenile period is more sensitive to the effects of exercise, thus resulting in lasting protection.

If exercise in the juvenile period is capable of long lasting upregulations in 5-HT_{1a}R expression, what are the mechanisms? Exercise-induced epigenetic modifications present a potential explanation. Epigenetics refers to molecular modifications to DNA that do not alter the genomic sequence, which can be environmentally mediated. These

include alterations in transcriptional modulators, DNA methylation, as well as histone acetylation and deacetylation. Histone modification, in particular, presents an intriguing potential mechanism. Histones are proteins around which DNA winds into units called nucleosomes and are an important factor in gene regulation. Histones can either be acetylated or deacetylated, increasing or decreasing gene expression, by adding or removing, respectively, an acetyl group to a lysine residue on the N-terminus of the histone tail (Magno, Steiner, & Caflisch, 2013; Potoyan & Papoian, 2012). 5-Hydroxytryptamine receptor 1A (HRT1a), the gene responsible for 5-HT_{1a}R expression, has been implicated to be susceptible to histone acetylation (de Moura, da Silva, et al., 2015; de Moura, Lazzari, et al., 2015). Further, 5-HT_{1a}R activity has also been shown to play a role in increasing global histone acetylation (Miyagawa, Tsuji, & Takeda, 2012; Tsuji, Miyagawa, & Takeda, 2014). Though we do not investigate epigenetic mechanisms directly, recent work from our lab investigating the effects of early life exercise on multiple physiological systems has shown that 3 weeks of exercise restricted to the juvenile period produces increases the short chain fatty acid (SCFA) butyrate, which is also an endogenous histone deacetylase (HDAC) inhibitor. As an HDAC inhibitor, butyrate could facilitate histone acetylation and thus increase gene expression. This butyrate-induced epigenetic mechanism could underlie how exercise initiated during the juvenile period induces long lasting stress protection.

The overall aim of the current study is to elucidate the neurobiological mechanisms by which early life exercise produces long lasting protection against the detrimental behavioral effects of IS. We first sought to determine if 6 weeks of exercise

initiated during the juvenile period produced a lasting increase in 5-HT_{1a}R expression. Juvenile rats were granted access to a running wheel for 6 weeks and their brains were extracted to measure mRNA expression levels of 5-HT_{1a}R either immediately or 25 days after exercise cessation. Next, in order to investigate which developmental period is important for producing these unique, long lasting effects, exercise was restricted to the juvenile period, by shortening the exercise time frame from 6 weeks to 3 weeks, to determine whether exercise during this period is sufficient in producing lasting protection against the behavioral consequences of stressor exposure. After 3 weeks of exercise, all rats remained sedentary for 15 days when they were exposed to IS and behaviorally tested for LH behaviors. Finally, epigenetic mechanisms, with an emphasis in histone acetylation, are discussed as a promising potential means by which early life exercise produces long lasting changes in gene expression. Feasible tools by which to measure both global and gene-specific histone acetylation are outlined for future research.

Materials and Methods

Rats

Juvenile (PND 24), male Fischer 344 rats were pair housed in standard Nalgene Plexiglas cages (45cm × 25.2cm × 14.7cm) with or without a running wheel. They were kept in a temperature (22°C) and humidity controlled environment and maintained on a 12:12 h light/ dark cycle (lights on from 0500-1700). The rats 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.

Experimental Design

Experiment 1: does exercise initiated in the juvenile period produce long lasting increases in 5-$HT_{1\alpha}$R mRNA expression? Juvenile rats (PND 24) were randomly assigned to either an exercise or sedentary group. Rats had access to a running wheel for 6 weeks. Immediately after exercise cessation, half of the rats from both sedentary and exercise groups were sacrificed and their brains extracted (0d). The remaining rats remained in their respective cages for 25 more days. During this time, the rats in the exercise condition had their wheels rendered immobile with metal stakes. Immediately following 25 days, these remaining rats were sacrificed and their brains extracted (25d). Brains from both cohorts were later processed for analysis of gene expression using *in situ* hybridization.

Experiment 2: does exercise restricted to the juvenile period produce long lasting stress protection against the behavioral consequences of stressor exposure? Juvenile

rats were randomly assigned to exercise or sedentary group for 3 weeks. After 3 weeks, all wheels were rendered immobile and all rats remained sedentary for 15 days. Immediately following 15 days, half of the rats in each group underwent IS (stress or no stress). The following day, all animals were behaviorally tested for LH behaviors.

Voluntary Wheel Running

Daily wheel revolutions were recorded digitally using Vital View software (Mini Matter, Bend, OR) and running distance was calculated (number of revolutions X wheel circumference (1.081m)).

In Situ Hybridization and Image Analysis

In experiment 1, tissue preparation and *in situ* hybridization procedures for analysis of gene expression followed previously established protocols (Greenwood, Foley, Day, et al., 2005; Greenwood et al., 2011). 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. DRN slices were collected for further analyses.

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. The riboprobe for 5-HT_{1a} was transcribed with the radioactive label Uridine 5'-

triphosphate UTP ([35S-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 H20 (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.

Exposure time for 5-HT_{1a} films was 2 weeks. Films were then developed (Konica Minolta Medical Imaging, model SRX-101A, Grand Rapids, MI, USA) in preparation of digital capture by use of computer-assisted optical densitometry (CCD camera, model XC-77; Sony, Tokyo, Japan). 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 a section of white matter. Within 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): DRN (rostral, -7.40mm to -7.64mm; mid, -7.80mm to -8.00mm; caudal, -8.30 to -8.50mm). 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.

Inescapable Stress

IS reliably produces LH behaviors in rats that resemble human symptoms of stress-related mood disorders, such as anxiety and depression. In this experiment, IS consisted of 100, 1.5 mA inescapable tail shocks administered at variable intervals (average ITI of 60 s) over a period of approximately 2 hours. 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. 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.

Behavioral Testing

24 h after IS, rats were placed in shuttle boxes (50.8cm × 25.4cm × 30.48cm, Coulbourn Instruments, Whitehall, PA) and assessed for shock-elicited freezing and shuttle box escape deficits within the same testing session, following previously established protocols(Greenwood et al., 2003). Rats were allowed 10 minutes of exploration upon placement in the novel environment, during which the rats were

scored every 10 seconds as freezing (no movement except for respiration) or not freezing. Rats then received 2 fixed ratio 1 (FR-1) foot shocks (0.1 mA, 60 s ITI). Once the rat crossed from one side of the shuttle box to the other, the shock would terminate. Latencies to cross were recorded. Immediately following FR-1 administration, shock-elicited freezing behavior was observed 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). The rats had to pass through the shuttle box door twice for shock termination. Again, 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 blind to treatment conditions. Testing occurred during the inactive cycle from 0800 to 1200, with each session lasting approximately 1 hour.

Statistical Analyses

Running distances were analyzed using repeated measures ANOVA. Levels of mRNA expression for 5-HT_{1a} were analyzed with ANOVA. Percent freezing average (over the 20 minute block) and FR-2 escape latencies were averaged into 5 blocks of 5 trials each and analyzed with ANOVAs. Butyrate levels were also analyzed with ANOVA. When a significant interaction was observed, Fisher protected least significant differences (F-PLSD) post hoc analyses were conducted. Results were significant when $p \leq 0.05$. Data are represented by means +/- SEM.

Results

6 weeks of early life exercise produces long lasting stress protection against the behavioral consequences of stressor exposure. Figure 1 depicts a summary of our previous experiment, where we found that 6 weeks of exercise in early life produces long lasting stress protection. Briefly, adult (PND 70) and juvenile (PND 24) rats had access to a running wheel or remained sedentary for 6 weeks. A cohort from each group was stressed and tested for LH behaviors immediately (0d), 15 days (15d), or 25 days (25d) after exercise cessation (Figure 1A). The adult runners were protected at the immediate time point, but were no longer protected at either 15d or 25d ($p<0.05$) as compared to controls. The juvenile runners, on the other hand were protected against the behavioral consequences of stressor exposure at all time points, thus demonstrating long lasting stress protection (Figure 1B and 1C).

Figure 1

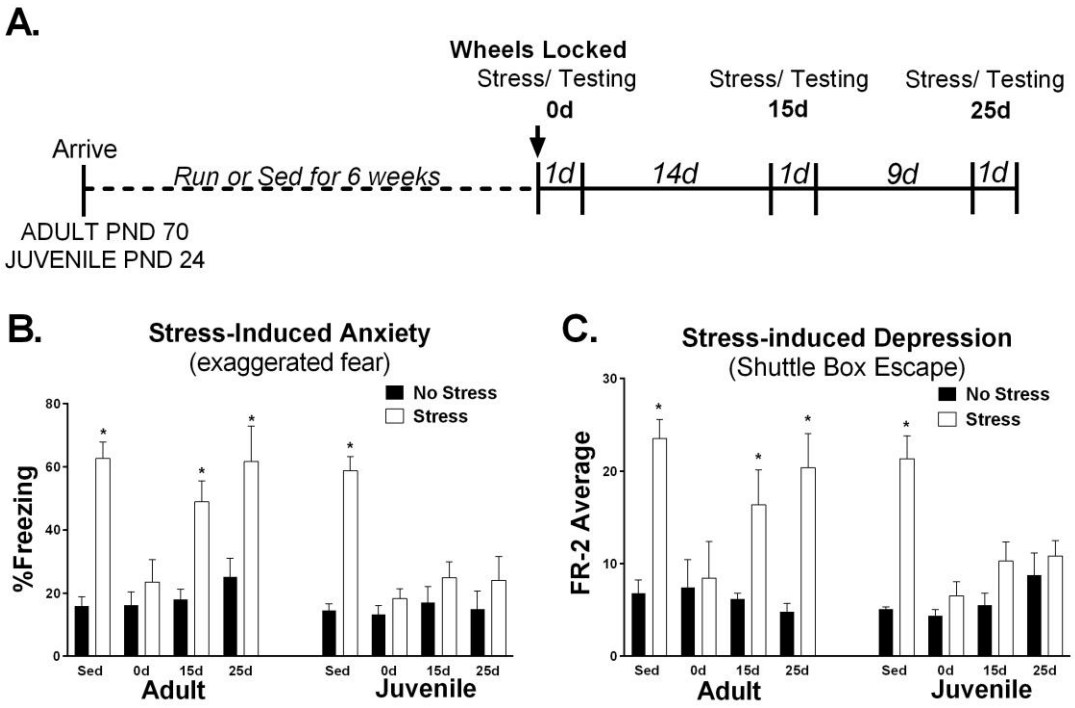

Figure 1: (A) Adult and juvenile rats had access to a running wheel or remained sedentary for 6 weeks. They were then exposed to IS and behaviorally tested for LH behaviors immediately (0d), 15 days (15d), or 25 days (25d) after exercise cessation. (B) Adults were transiently protected against IS-induced exaggerated fear while juveniles were persistently protected. (C) Adults were transiently protected against IS-induced shuttle box escape deficits while the juveniles were persistently protected. * denotes $p<0.05$

6 weeks of early life exercise produces long lasting increases in 5-HT_{1a}R mRNA levels. In the current experiment, we investigated the neurobiological alterations that make this long lasting stress resistance possible. We sought to determine whether 6 weeks of exercise in early life produces accompanying lasting increases in 5-HT_{1a}R mRNA expression. Figure 2 depicts the experimental design of this experiment (Figure 2A) where after 6 weeks of exercise or sedentary conditions brains were collected and analyzed for 5-HT_{1a}R mRNA both immediately and 25 days (25d) after exercise cessation. In Figure 2B, the running distance is shown. Juvenile rats increased running distance until week 4, where it plateaued. 5-HT_{1a}R mRNA expression levels for both sedentary controls and juvenile runners is shown in Figure 3A. There was a main effect of exercise ($F_{1, 37}$=4.230, p=0.0468) showing that juvenile runners had increased levels of 5-HT_{1a}R mRNA within the rostral DRN, as compared to controls. A representative image of the region quantified for 5-HT_{1a}R mRNA level analysis is also shown (Figure 2D).

Figure 2

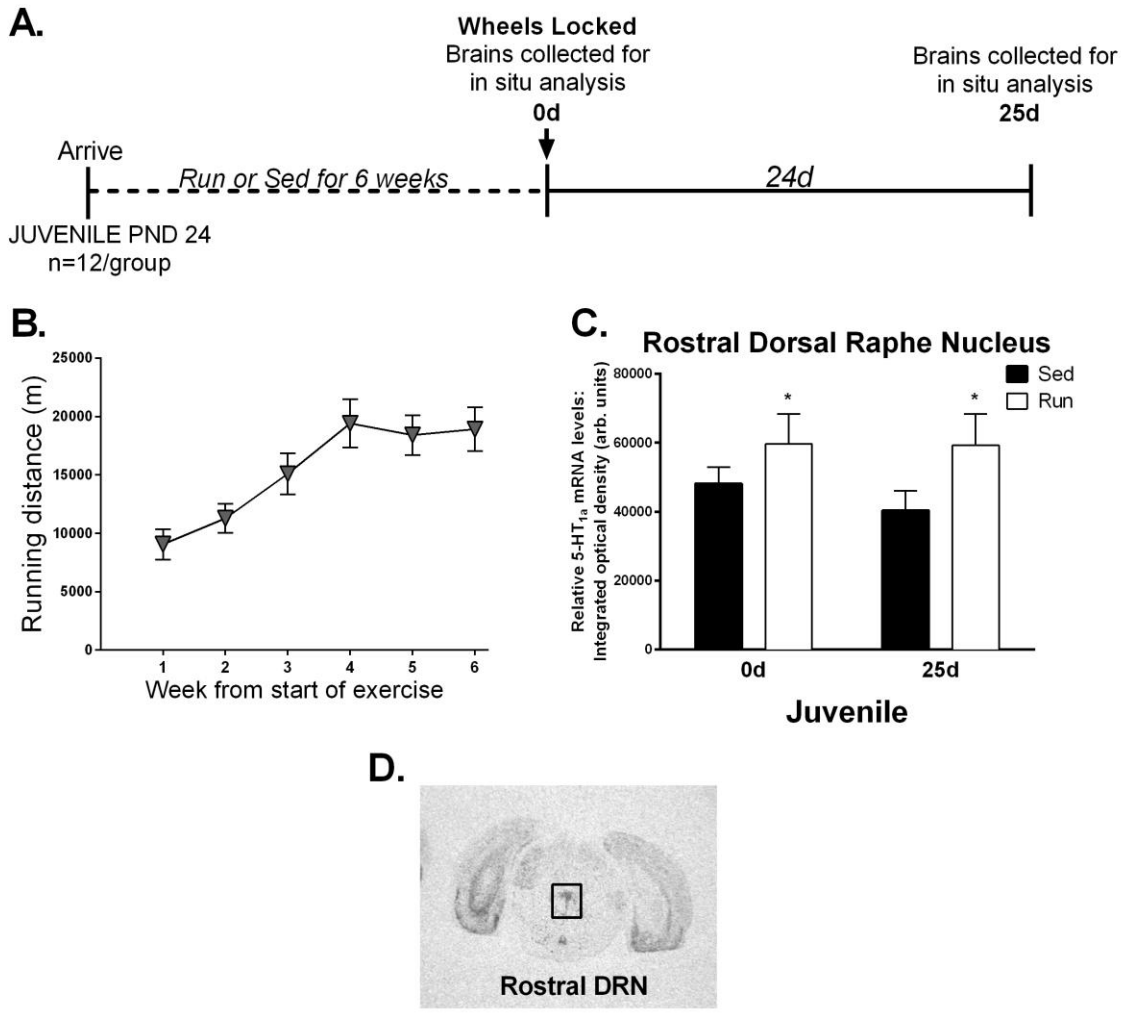

Figure 2: (A) Experimental design to determine whether 6 weeks of early life exercise produces long lasting alterations in 5-HT_{1a}R mRNA levels. (B) Running distance. (C) Juvenile runners had increased levels of 5-HT_{1a}R mRNA levels. (D) An autoradiographic image of the rostral DRN showing relative levels of 5-HT_{1a}R. * denotes $p<0.05$

Exercise restricted to the juvenile period is sufficient in producing long lasting stress protection. In the second experiment, we sought to investigate whether exercise during only the juvenile period was sufficient in protecting against the behavioral consequences of stressor exposure. We therefore restricted exercise to the juvenile period by shortening the exercise duration from 6 weeks to 3 weeks then IS and behaviorally tested all animals 15 days (15d) after exercise cessation (Figure 3A). Juvenile runners were protected against stress induced LH behaviors. There was a main effect of exercise ($F_{1, 43}$=7.807, p=0.0077) and stress ($F_{1, 43}$=17.874, p=0.0001) on exaggerated freezing (Figure 3B). There was also a main effect of exercise ($F_{1, 43}$=6.284, p=0.0160) and stress ($F_{1, 43}$=13.321, p=0.0007) in shuttle box escape deficits, as well as a significant interaction between exercise and stress ($F_{1, 43}$=5.631, p=0.0222; Figure 3C).

Figure 3

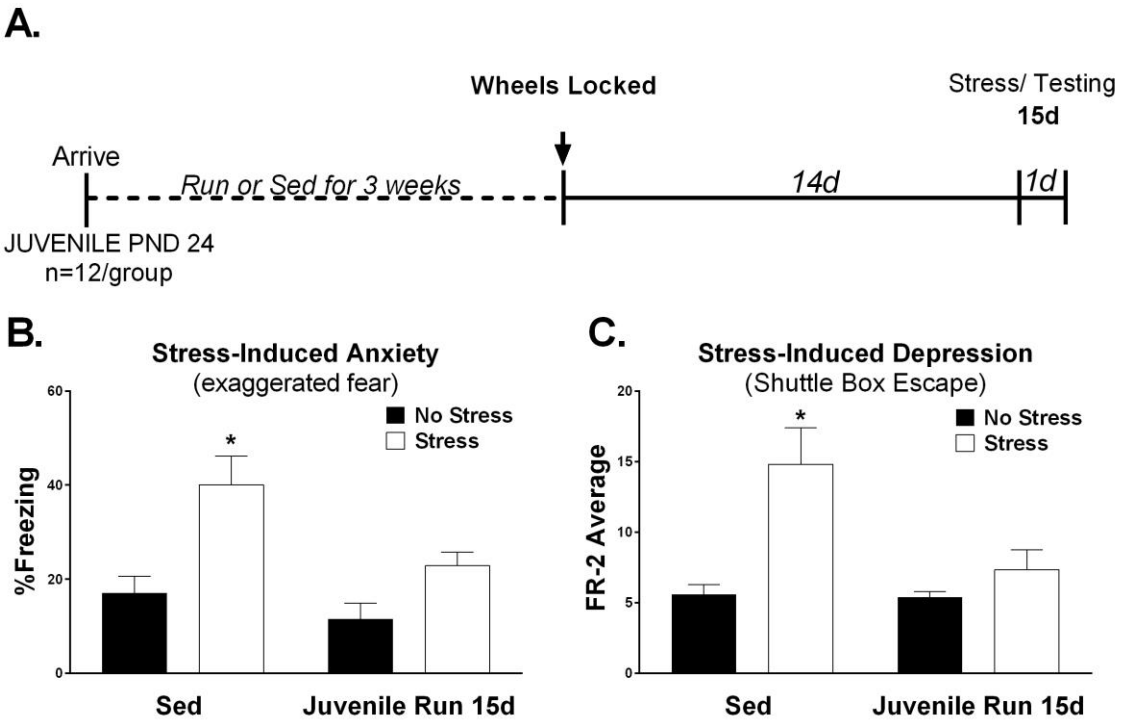

Figure 3: (A) Experimental design to determine if exercise during the juvenile period is sufficient in producing long lasting stress protection. (B) Runners were protected against exaggerated freezing. (C) Runners were protected against shuttle box escape deficits. * denotes $p<0.05$

3 weeks of exercise during the juvenile period increases levels of butyrate, an endogenous HDAC inhibitor. Figure 4 depicts data demonstrating that three weeks of exercise in the juvenile period increases fecal concentrations of the HDAC inhibitor butyrate. Briefly, juvenile rats were given access to a running wheel or remained sedentary for 3 weeks at which point fecal samples were collected and analyzed for levels of butyrate (Figure 4A). There was a main effect of exercise ($F_{1, 11}$=8.827, p=0.0157) indicating that juvenile runners had increased levels of butyrate as compared to controls.

Figure 4

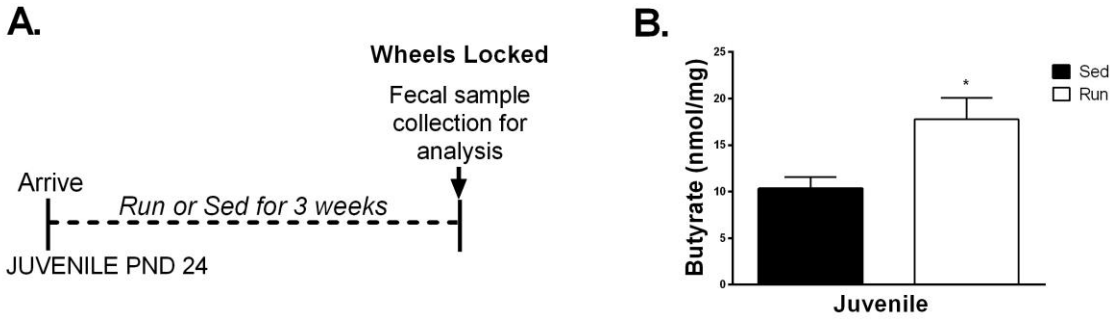

Figure 4: (A) Juvenile rats had access to a running wheel or remained sedentary for 3 weeks. After 3 weeks, the wheels were locked and fecal samples were collected for analysis. (B) Juvenile runners have increased levels of butyrate. * denotes $p<0.05$

Discussion

The protective effects of exercise against the behavioral consequences of stressor exposure are age dependent. Adult runner rats are temporarily protected compared to juvenile runners, who still exhibit unwavering protection 25 days following cessation of exercise. The current study adds to our understanding of how exercise impacts neurobiology in order to produce these lasting stress protective effects. In our first experiment, juvenile rats that ran for 6 weeks displayed increased levels of 5-HT_{1a}R mRNA expression both immediately following exercise cessation as well as 25 days following exercise cessation. These data indicate that the lasting increases in 5-HT_{1a}R mRNA follow the same pattern as the behavioral stress protective effects, suggesting that alterations in 5-HT_{1a}R expression may underlie long lasting stress protection. However, further research is needed to determine whether these increases in 5-HT_{1a}R mRNA are truly a causal mechanism.

Our second experiment sought to determine whether a shorter exercise regimen during the juvenile period specifically is sufficient to produce long lasting protection against the behavioral consequences of stressor exposure, and therefore, refine our possibilities of neurobiological mechanisms underlying this effect. We restricted exercise to 3 weeks within the juvenile period and found that juvenile runner rats were still protected against stress-induced LH behaviors 15 days after exercise cessation. This is of particular note since we've shown that adult rats given 3 weeks of exercise are not protected against LH behaviors (Greenwood, Foley, Burhans, et al., 2005). Thus, adult rats not only have to continually exercise in order to reap the stress protective effects, but they must run twice as long to even induce protection. This further

suggests that there must be something unique about exercise during juvenile period enabling production of lasting stress protection.

The mechanisms underlying these long term changes in gene expression are currently unknown. Data from our second experiment suggest that 3 weeks of exercise restricted to the juvenile period may also produce long lasting changes in gene expression, though this was not explicitly determined. The ability of early life exercise to produce lasting stress protection may be attributed to the sensitive and highly plastic nature of the young, developing brain during the uniquely labile juvenile period (Andersen, 2003). During early life development there is an overabundance of plasticity related proteins and growth factors. As a result, there is an explosive amount of new connections (synapses) and rapid formation, as well as peaks in density, of neural circuitry. Because of this enhanced plasticity, external stimuli and experiences can more efficiently impact the brain. For example, we show that 6 weeks of exercise initiated during the juvenile period produces long lasting alterations to the 5-HT circuit and others have shown also that the juvenile period is sensitive to 5-HT circuit modulation (Booij et al., 2015), suggesting that exercise occurs during a developmentally sensitive time for 5-HT_{1a}R maturation in the DRN. In another study, 5-HT_{1a} expression levels were shown to double within the DRN during the juvenile period (Sidor et al., 2010). This continued development throughout the juvenile period could therefore leave this receptor population within the DRN sensitive to exercise-induced alteration during this time, potentially underlying persistent protection against stress that lasts throughout the organism's lifespan.

Given that we observe lasting increases in gene expression, epigenetic changes present a promising potential mechanism by which this lasting imprint occurs. Increasing or decreasing

levels of transcriptional modulators as well as DNA methylation can either enhance or repress gene expression. There are several transcriptional modulators of HRT1a, including nuclear deformed epidermal autoregulatory (NUDR) and 5' repressor element-1 under dual repression binding protein (Freud-1), both potent modulators that have also been shown to be affected by stressor exposure (Ou et al., 2003; Szewczyk et al., 2014). Alternatively, the genomic sequence of HRT1a could by methylated to affect 5-HT_{1a} expression levels (Gudsnuk & Champagne, 2012; Le Francois et al., 2015; Mychasiuk, Muhammad, & Kolb, 2016). A more intriguing mechanism, however, is histone acetylation.

In the current manuscript, we displayed data from another experiment that examined how early life exercise altered microbial metabolites within the gut. Interestingly, 3 weeks of exercise initiated during the juvenile period increased endogenous butyrate. Butyrate is a short chain fatty acid (SCFA) produced by fermentation of various dietary polysaccharides by microbes within the gut and is capable of crossing the blood-brain barrier (Braniste et al., 2014; De Vadder et al., 2014; Donohoe et al., 2011). As an HDAC inhibitor, butyrate facilitates histone acetylation (Candido, Reeves, & Davie, 1978; Sealy & Chalkley, 1978), leading to an increase in gene transcription for various genes, including BDNF (Intlekofer et al., 2013) and potentially HRT1a. In fact, it has recently been shown that treatment of sodium butyrate can alter the amount of 5-HT expressed within the hippocampus (Sun et al., 2016) as well as expression levels of 5-HT_{1a}R (Zhu, Huang, Xu, Niu, & Zhou, 2009), though the precise mechanism by which this occurs is currently unknown. Therefore, it is possible that exercise during the juvenile period produces an increase in butyrate which, acting as an HDAC inhibitor, leads to increased

global histone acetylation which may benefit and increase HRT1a transcription and thus lead to increased expression of 5-HT1aR.

HDAC inhibitor-induced alterations in 5-HT_{1a}R expression are possible because HRT1a is epigenetically modifiable. Indeed, others have also shown that adaptive manipulations, such as enriched environments, complex housing or access to a running wheel can lead to increases in global histone acetylation (Fischer, Sananbenesi, Wang, Dobbin, & Tsai, 2007; Gudsnuk & Champagne, 2012) as well as transcriptional expression of HTR1a (de Moura, Lazzari, et al., 2015). More recently, this increase in HRT1a transcription has been attributed to an increase in global acetylated histone H4 within the hippocampus (de Moura, da Silva, et al., 2015). Though studies have not examined the DRN specifically, these studies demonstrate the epigenetic modifiability of HRT1a, suggesting that an increase in global histone acetylation within the DRN could potentially benefit HRT1a and therefore allow for long lasting increases in 5-HT_{1a}R expression within the region.

While examining the potential epigenetic mechanisms by which long lasting stress protection is produced, it is interesting to note that activation of 5-HT_{1a}R can also lead to further epigenetic modifications in the surrounding brain region. A study conducted within the hippocampus demonstrated that a 5-HT_{1a} agonist led to global hyperacetylation of histone H3 (Miyagawa et al., 2012). Though this study was unable to localize this histone acetylation to any specific genes, it is not too drastic to suggest that HRT1a may benefit from this histone acetylation, further increasing expression of 5-HT_{1a}R. Another study showed that 5-HT_{1a}R activation also alters the expression of a multitude of different genes (Tsuji et al., 2014), including the downregulation of several HDAC genes. This downregulation would decrease the

amount of deacetylase enzymes available, thus inhibiting histone deacetylation and increasing gene expression. It therefore seems that 5-HT_{1a}R activation not only limits the typical LH behavioral response but also produces downstream epigenetic modifications potentially creating a positive feedback loop on HRT1a histone acetylation and further 5-HT_{1a}R expression.

However, interaction between histone acetylation, HRT1a, and 5-HT_{1a}R remains unclear as it is difficult to colocalize histone modifications with specific genes. When examining global histone acetylation, the total histone acetylation within a particular brain region, it is common to use techniques like Western blot (WB) analysis or immunohistochemistry (IHC). In one study examining butyrate and its antidepressant-like role in the hippocampus (Han, Sung, Chung, & Kwon, 2014), a WB was performed by tissue dissection, cell lysis, and separation of proteins by gel electrophoresis. Those proteins were then transferred to a membrane and immunoblotted with antibodies against histone acetyl-histone H3. After incubation with a secondary antibody, the membranes were visualized with a solution that produces chemiluminescent signal that can be captured and quantified to measure specific protein (acetylation) levels. They then utilized IHC on individual slices of hippocampal tissue by incubating them with an acetylated histone H3 antibody. The cells that were then immunoreactive to that antibody were counted using microscopy. These techniques revealed a global increase of histone acetylation in the hippocampus due to butyrate action.

A more useful method for detecting gene specific histone acetylation involves chromatin immunoprecipitation (ChIP) assay followed by polymerase chain reaction (PCR), reverse transcription-quantitative PCR (RT-qPCR), DNA microarrays, or DNA sequencing (Collas, 2010). In a ChIP assay, chromatin is extracted from tissue, the DNA and proteins are crosslinked and

subsequently fragmented. These fragments are then immunoprecipitated with specific antibodies that target particular proteins into complexes that are purified until only DNA fragments remain, which can be easily identified by several different assays. This method was used by Intlekofer et al. (2013) in their examination of exercise and sodium butyrate on transcriptional regulation of brain derived neurotrophic factor (BDNF). Hippocampal tissue was immunoprecipitated with antibodies against acetyl histone H4 and the resulting DNA fragments were identified by RT-qPCR. First, BDNF RNA was transcribed into complementary DNA (cDNA) by reverse transcriptase to create a primer for the amplification reaction. Then the resulting samples from ChIP and the BDNF primer were added together in qPCR. Levels of BDNF DNA amplification were determined by a change in fluorescence measurements as compared to controls. This combined method isolated all DNA fragments associated with acetylated histones and then identified at least a portion of those fragments as the BDNF gene, demonstrating that butyrate increased BDNF gene expression by histone acetylation.

This combined ChIP and RT-qPCR method could easily be modified to examine acetylation of other genes of interest, including HRT1a, given the appropriate primers, and help to more completely understand the underlying mechanism of long lasting stress protection produced by exercise initiated during the juvenile period. Studies utilizing these methods could provide the missing link between exercise and HRT1a histone acetylation and 5-HT_{1a}R expression upregulation as well as 5-HT_{1a}R activation and further epigenetic modifications, thus determining the validity of the epigenetically-induced positive feedback loop on long lasting HRT1a histone modification and 5-TH_{1a}R expression.

Conclusion

6 weeks of early life exercise produces a persistent increase in 5-HT_{1a} mRNA and 3 weeks of exercise restricted to the juvenile period is sufficient in producing long lasting stress protection. These data suggest that (1) exercise initiated during the juvenile period produces long lasting stress protection likely subserved by alterations in the 5-HT circuitry and (2) juveniles require half the amount of exercise as adults to not only be protected, but be protected long after exercise cessation. Because the juvenile period is uniquely characterized by a highly plastic brain and increased sensitivity to 5-HT circuitry modulation, it is possible that epigenetic modifications underlie long lasting stress protection. Recent work from our lab shows an increase of butyrate, an HDAC inhibitor, after 3 weeks of exercise initiated in the juvenile period. This increase in butyrate could lead to increased global histone acetylation, which may benefit genes important in the stress response, like HRT1a. If the histones associated with HRT1a are acetylated due to HDAC inhibitor activity, then 5-HT_{1a}R expression would increase and subsequent 5-HT_{1a}R activity would produce even more histone acetylation further increasing transcription of HRT1a. In this way, there may be a positive feedback loop where an increase in butyrate may jumpstart an epigenetic mechanism (histone acetylation) resulting in long lasting transcriptional alteration of HRT1a leading to lasting increased expression of 5-HT1aR. Though mechanism has not yet been fully investigated, combining research methods like ChIP and RT-qPCR provides a promising tool for the examination of gene specific histone acetylation. Future investigations could easily examine HRT1a histone acetylation after 3 weeks of exercise during the juvenile period or after injection of butyrate to better understand the validity of a potential epigenetic positive feedback loop on HRT1a. This

thesis begins the characterization of the mechanism underlying long lasting stress protection in physically active juvenile rats and gives insight as to which methodological tools could be used in future investigations of potential epigenetic mechanisms that may underlie exercise-induced long lasting stress protection.

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