The Effects of Early-Life Exercise on Neurotrophic Factor Expression Throughout the Lifespan

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Abstract

Early life experiences play a pivotal role in both brain functioning and behaviors of the adult. Exercise has beneficial effects that may influence the developing brain differently than the mature brain. While the dependence of brain development on early life experiences and the beneficial effects of exercise are both well established, the effects of exercise as an early life experience are relatively unknown. The purpose of this study is to quantify the mRNA expression of two growth factors, BDNF and IGF-1, in various brain regions of two groups of animals: animals that were allowed access to running wheels for 6 weeks beginning early in life (P24; early life exercise, ELE) or during adulthood (P70; adult exercise, AE). These animals were compared to their sedentary controls, both early life (P24; early life sedentary, ELS) and adult (P70; adult sedentary, AS). To examine the possibility of persistent exercise-induced effects, the animals were split into two groups: (1) effects immediate following 6 weeks of exercise and (2) effects 25 days following the cessation of exercise. BDNF mRNA levels were quantified in the hippocampus, amygdala, and motor cortex. . In the dentate gyrus, there was a significant increase in BDNF mRNA immediately following the cessation of exercise in both ELE and AE compared to sedentary controls. 25 days following the cessation of exercise, AE BDNF levels were back to baseline while ELE rats expressed a persistent increase in BDNF mRNA. In nearly every brain region, BDNF mRNA levels were higher in the ELE and ELS compared AE and AS, and decreased with age. These data support the hypothesis that early life exercise has persistent beneficial effects.

Introduction

Physical exercise has benefits ranging from enhanced quality of life to improvements in overall health and cognition (Dishman et al., 2006). Exercise, for example, improves various learning and memory processes including hippocampal-dependent spatial learning (Anderson et al., 2000; Ang et al.,

2006; Fordyce and Farrar, 1991; Fordyce and Farrar, 1991; O'Callaghan et al., 2007), non-hippocampal-dependent forms of learning (Hopkins et al., 2011; O'Callaghan et al., 2007), and contextual fear memory (Baruch et al., 2004; Greenwood et al., 2009). These types of learning and memory utilize different brain regions, thus the effects of exercise are not localized to a particular region but rather seem to generalize to many functions and circuits brain-wide. Additionally, exercise can reduce the incidence and severity of stress-related psychiatric disorders such as depression and anxiety (Greenwood and Fleshner, 2013) and can produce anti-depressant-like effects (Greenwood et al., 2007). The antidepressant and anxiolytic effects of exercise have been well established in both animal models (Duman et al., 2008; Fulk et al., 2004; Greenwood et al., 2003; Salim et al., 2010) and humans (Mather et al., 2002; Rethorst et al., 2009; Wipfli et al., 2008). We have previously reported that 6 weeks of voluntary wheel running, for example, can prevent the depression- and anxiety-like behavioral consequences of exposure to uncontrollable stress (Greenwood et al., 2007). While the benefits of exercise are clearly well known, the neurobiological mechanisms for these benefits are not well established.

Recent observations suggest that increasing physical activity during early, critical periods of development could be particularly beneficial in that it could produce lasting, stable benefits across the lifespan. Hopkins et al. (2011), for example, investigated the effects of early life exercise compared to exercise initiated in adulthood on performance in a non-hippocampal dependent memory task. Consistent across age groups, 4 weeks of wheel running led to enhanced performance in a novel-object recognition memory task. Two weeks following the cessation of exercise, the memory enhancements were absent in rats that started exercising as adults. The memory enhancing effect, however, was still apparent for four weeks after the cessation of exercise if exercise was initiated during early life (Hopkins et al., 2011). Spatial, hippocampus-dependent learning and memory tasks show a similar trend of long-term effects of exercise initiated in early life. Compared to sedentary controls, rats subjected to treadmill

running from P21-P60 showed enhanced performance in a water maze immediately following exercise and significantly better long-term memory of the task, as signified by a "re-test" of the maze 30 days later (Gomes da Silva et al., 2012). The ability of early life exercise to increase the persistence of exercise benefits may not be restricted to learning and memory, as similar observations have been made with respect to the anxiolytic and antidepressant effects of exercise. If exercise is initiated immediately after puberty (~P49), these effects last between 15 and 25 days (Greenwood et al., 2012). However, when exercise is initiated during early life (defined as the period immediately post weaning and before puberty[1]; P24; early life exercise, ELE), the anxiolytic and antidepressant effects of exercise are still apparent and show no signs of attenuation 25 days after cessation of exercise (Mika et al., unpublished observations). ELE may interact with a critical period in development that is characterized by ubiquitous neuronal plasticity, during which immense pruning occurs (for review see (Hensch, 2005). These neuronal pruning processes are activity dependent. During this time, important structural modifications establish neural circuitry for the mature brain. Thus exercise may have different long-term effects on the developing brain than the developed brain. The mechanisms by which this occurs is elusive but likely involves molecules that are important for development and are modulated by exercise.

Growth factors produce benefits similar to those produced by exercise including increased synaptic plasticity, improvements in learning and memory tasks, and antidepressant effects (Groves, 2007; Siuciak et al., 1997). The most abundantly expressed growth factor in the brain, brain derived neurotrophic factor (BDNF), is critically important for neuronal health, promoting processes such as cell growth and proliferation. BDNF has both neuroprotective traits and induces anti-apoptotic

[1] It should be noted that terminology for this particular period is inconsistent; it is referred to as "juvenile" or "adolescent" periods seemingly interchangeably. For our purposes, we are concentrating on the period post-weaning, pre-puberty for "early life"

signaling cascades within the cell (Hetman et al., 1999). A second growth factor, insulin-like growth factor-1 (IGF-1), is structurally similar to insulin and has traits similar to BDNF including promoting cell development, proliferation, and division. IGF-1 is also a cytoprotectant, a factor that protects the cell from damage. For example, IGF-1 activates pathways that are directionally opposite to apoptotic pathways that can be activated in mesangial cells exposed to high levels of glucose; thus maintaining mitochondrial integrity and preventing apoptosis (Kang et al., 2003). Although mesangial cells are functionally different from neurons, these cells can give insight to the signaling pathway of IGF-1. Through various signaling pathways IGF-1 and BDNF signaling produces beneficial effects.

Exercise increases growth factors and this increase has been linked to exercise-induced effects. Both BDNF (Adlard et al., 2004; Neeper et al., 1995; Neeper et al., 1996) and IGF-1 (Ding et al., 2006) mRNA levels are elevated by exercise. Although we have reported that the exercise-induced reduction of the depression-like behavior, learned helplessness, as tested by shuttle box escape is independent of BDNF levels in the hippocampus (Greenwood et al., 2007), other assays of anti-depressant effects of exercise are abolished if BDNF signaling is inhibited. For example, the forced swim test (FST) is a method used to test the efficacy of antidepressant drugs. Exercise improves FST performance; however, rats with a heterozygous deletion of the BDNF gene show no effect of exercise on FST performance (Duman et al., 2008). BDNF is also critically important for the learning and memory enhancements produced by exercise. For example, BDNF mediates the exercise-induced enhancements in the Morris water maze such that inhibition of BDNF in the hippocampus prior to running abolished running-induced enhancements (Vaynman et al., 2004). IGF-1 mRNA is increased with exercise and interacts with exercise-induced BDNF. Indeed, IGF-1 has been causally linked to the neuroprotective effects induced by exercise, including the exercise-induced BDNF increases. (Carro et al., 2000). These data suggest that growth factors are necessary for the learning and memory enhancing and antidepressant-like effects of exercise.

Taken together, the fact that exercise increases growth factors and growth factors have similar beneficial effects as exercise suggests that enhanced neurotrophic support may be mediating the beneficial effects of exercise. Since the pre-pubertal brain is particularly sensitive to environmental manipulations, long-lasting exercise-induced effects could be due to long-lasting increases in growth factors that persist following the termination of exercise.

The purpose of this study was to determine whether exercise-induced increases in growth factor (BNDF, IGF-1) mRNA levels in brain regions implicated in learning and memory as well as stress-related psychiatric disorders (hippocampus and amygdala) persisted longer following cessation of exercise if exercise was initiated during early life relative to adulthood. Animals were allowed to run for 6 weeks initiated either in early life (P24; early life exercise, ELE) or adulthood (P70; adult exercise, AE) and compared to early life sedentary (P24; ELS) and adult sedentary (P70; AS) controls, mRNA levels were investigated using *in situ* hybridization. We hypothesize that the exercise-induced increase in BDNF and IGF-1 mRNA typically observed immediately after 6 weeks of voluntary exercise will persist longer following the termination of exercise in ELE animals. These data will add to our understanding of how exercise early in life can set a foundation for a healthy life. The results could provide insight into the mechanism underlying the longer-lasting exercise effects when exercise is initiated at a young age.

Materials and Methods

Animals

Adult (appx. P70; n=24) and early-life (appx. P24; n=24) male, Fischer F344 rats were housed in a temperature (22°C) and humidity-controlled environment and were maintained on a 12:12 h light/dark cycle (lights on 06:00-18:00). All rats were allowed access to voluntary running wheels immediately upon arrival and were pair-housed in Nalgene Plexiglas cages (45 x 25.2 x 14.7 cm). Rats were weighed

weekly and given *ad libitum* access to food and water. Care was taken to minimize animal discomfort during all procedures. All experimental protocols were approved by the University of Colorado Animal Care and Use Committee.

Experimental Design

Animals were P24 and P70 upon arrival and those within the exercise groups were given access to a running wheel immediately upon arrival. Generally animals are given a week acclimatization period; however, due to the age of our animals, the acclimatization period was not used within this paradigm. Wheels were locked with a metal stake 6 weeks after initial wheel access and 0-day-timepoint (0D) animals were sacrificed; 25 days later the remaining animals were sacrificed at the 25-day-timepoint (25D). Animals were pair housed within groups throughout the course of the study. N=48 total; 6 per group. Groups are as follows: early life exercise (P24; ELE), early life sedentary (P24; ELS), adult exercise (AE), and adult sedentary (AS). Half of the animals in each group were sacrificed immediately following the cessation of exercise (0D timepoint) and the remaining rats were sacrificed 25 days following the cessation of exercise (25D timepoint).

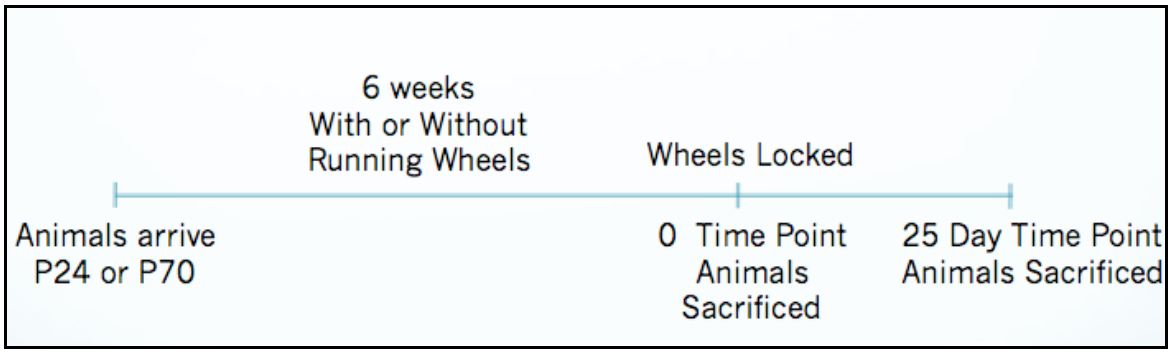

Figure1

Experimental design. Adult or early-life animals were double housed either with our without running wheels for 6 weeks. Animals in the RUN group were given access to running wheels immediately upon

arrival. Wheels were locked at the end of 6 weeks, at which time animals in the 0D cohort were sacrificed. 25 days following locking the wheels, animals in the 25D cohort were sacrificed.

Voluntary wheel running

Rats were randomly assigned to either remain sedentary in standard cages with no wheels (Sedentary condition; SED) or were housed in standard cages equipped with running wheels (Mini Mitter, Bend, OR; Run condition; RUN). Rats in the run condition were allowed voluntary access to wheels for 6 weeks, after which wheels were locked with metal stakes throughout the remainder of the experiment (25 day time point; 25D) or sacrificed via rapid decapitation (8AM-11AM) immediately after 6 weeks of RUN or SED conditions (0 day timepoint; 0D). Daily wheel revolutions were recorded digitally using Vital View software (Mini Mitter) and distance was calculated by multiplying number of revolutions by wheel circumference (1.081 m).

Tissue Processing

Brains were extracted and flash frozen in isopentane cooled with dry ice (-20°C; 4 minutes). Brains were stored at -80°C prior to being sectioned at a thickness of 10 μm with a cryostat. Slicing occurred at -21°C , and rostral-caudal sections of the hippocampus and amygdala were collected and thaw-mounted onto FisherBrand Colorfrost®Plus slides (Fisher Scientific Company LLC, Denver, CO, USA) . Tissue sections were stored at -80°C prior to use in single-label radioactive *in situ* hybridization.

Preparation of cDNA Probes

RNA Extraction

RNA, for preparation of the IGF-1 clone, was isolated using the SV Total RNA Isolation Kit (Promega Corporation. Madison, WI). Briefly, RNA was extracted from the frontal cortex and hippocampus of a sedentary, adult rat. The rat was sacrificed via rapid decapitation and the brain was extracted. The

prefrontal cortex and hippocampus were microdissected and immediately homogenized in RNA Lysis Buffer on ice. RNA Lysis Buffer allows RNA to be released into solution, inactivates ribonucleases, and precipitates proteins. The precipitated proteins were pelleted via centrifugation (14,000 RPM, 10 min) followed by multiple washes to rid the sample of residual cellular debris. Subsequently, the RNA was selectively precipitated in an ethanol wash and bound to a spin basket. DNase I was introduced to digest any genomic DNA, after which the RNA was washed from the spin basket, aliquoted based on brain region, and stored at -80°C. RNA concentration was verified with the Qubit RNA Assay Kit, which utilized fluorescence to measure the concentration of RNA. In a 0.5 mL PCR tube, light-sensitive Qubit RNA Reagent was diluted (1:200) in Qubit RNA Buffer to yield Qubit "working solution". RNA samples were then diluted (1:200) in Qubit working solution. All RNA concentrations were confirmed to be between 250 and 300 ng/mL, after which the RNA was returned to -80°C. RNA quantification required thawing the RNA sample on ice; however, freeze thawing can be disruptive to the RNA integrity. To avoid using degraded or damaged RNA in an assay, only one of the RNA aliquots (per brain region) was thawed and quantified. It was presumed that the concentration of all aliquots were equal, thus the sample that was quantified represented the whole RNA sample and was not used in subsequent assays.

Polymerase Chain Reaction

Easy-oligo (100 μM concentration) DNA primers (Sigma-Aldrich, St. Louis, MO, USA) from primer sequences derived from the National Center for Biotechnical Information (NCBI) database were used to develop each probe. The primers for the IGF-1 probe were: forward ACGTCACCGCAAGATCCTTT, reverse TGCTTTCGAGGAGGCCAAAT; expected product length was 566 basepairs. Polymerase Chain Reaction (PCR) was carried out with the SuperScript One-Step RT-PCR with Platinum Taq kit (Sigma-Aldrich, St. Louis, MO, USA). Primers were combined with previously extracted RNA (approximately 280 ng/mL), 2X

reaction mix (buffer containing 0.4 mM of each dNTP, 2.4 mM $MgSO_4$) and Taq polymerase in PCR tubes (0.2 mL) and subjected to a thermocycler. Cycling conditions were set according to the manufacturer's suggestions, as follows: cDNA synthesis and pre-denaturation (1 cycle of 55°C for 15-30 s, 94°C for 2 min), PCR amplification (40 cycles of denaturation, 94°C for 15 s; annealing, 60°C for 30 s; extension, 68°C for 1 min/kb), and final extension (1 cycle of 72°C for 5-10 min). PCR product size and amplification was confirmed via gel electrophoresis.

Gel Electrophoresis

Agarose gel (1% agarose gel in 0.5X TBE) was heated and stirred until homogenous, ethidium bromide was added and poured into a gel tray. When solidified, cold (4°C) 0.5X TBE was poured over gel. PCR products, as well as a 1Kb DNA ladder (New England Biolabs, Ipswich, MA, USA) were combined with 0.5X TBE and loading dye (New England Biolabs, Ipswitch, MA, USA) and loaded into the solidified gel, which was run for 1 hour at 100 volts. Bands were visualized using ultraviolet light.

Ligation and Transformation

After successful amplification of the sequence of interest, PCR products were ligated and transformed with the StrataClone PCR Cloning Kit (Agilent Technologies, La Jolla, CA, USA) following the manufacturers suggestions. Ligation is the process of inserting the PCR product into engineered vector arms. The PCR product was ligated into the vector by briefly (5 min) incubating with Strataclone Cloning Buffer and Strataclone Vector Mix ampicillin before placing on ice. During ligation, the uricil overhang engineered into the vector binds to the adenosine overhang on the PCR product generated by Taq polymerase, ligating the PCR product into the vector arms. Subsequently, the vector arms are fused via Cre recombinase to form circular DNA (vector). Transformation, the process by which the vector is taken

up by competent cells, occurred immediately following ligation. The vector was added to a thawed tube of competent cells, incubated on ice (20 min), heat shocked (42°C for 45s) and placed on ice (2 min). Pre-warmed LB broth was added to the mixture and incubated for 1 hour (37°C) with agitation. During this time, pre-prepared 2% X-gal (0.2 g 5-bromo-4-chloro-3-inodyly-β-galactopyranoside (X-gal), 10 mL dimethylformamide (DMF)) was spread (40 μl per plate) on pre-prepared LB-ampicillin plates. Using sterile technique, cells were spread on plates with a cell spreader at two dilutions (5 μl and 100 μl) and incubated overnight (37°C). The next day, mini preps were prepared: white colonies were inoculated in a mini-prep (LB broth, ampicillin, colony) and incubated (37°C) for 5 hours then transferred to a fresh mini-prep (LB broth, ampicillin) and incubated overnight. The following day, the mini-prep was used to create a glycerol stock (stored at -80°C) for long-term plasmid storage as well as prepared for *in situ* hybridization. The IGF-1 DNA was successfully cloned and prepared; however, due to time constraints only the BDNF *in situ* hybridization was completed, as detailed below.

Single Label Radioactive In-Situ Hybridization

In situ hybridization was conducted following previously published protocols (Day and Akil, 1996, (Greenwood et al., 2011; Greenwood et al., 2012; Greenwood et al., 2003). Brain tissue slides were fixed in 4% paraformaldehyde for one hour, 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 grade ethanol. A previously prepared BDNF (Accession #: NM_012513, 750 bp) complementary DNA probe was transcribed with ATP, CTP, GTP and S^{35} UTP (radioactive tag) to create a riboprobe. After verification of radioactive labeling, the riboprobe was mixed with 50% hybridization buffer comprised of 50% high-grade formamide, 1-% dextran sulfate, 3X SSC, 1X Denhardt's solution, 0.2 mg/mL yeast tRNA, and 0.05 M sodium phosphate (pH 7.4). The riboprobe and hybridization buffer mixture was applied

directly to slides containing sections of PFC and hippocampus. Slides were incubated overnight at 55°C in a humid chamber. The next day, slides were washed 3 times in 2X SSC and incubated for 1 hour in RNase A (200 μg/mL) at 37°C to degrade unbound RNA. Subsequently, tissue was rinsed in graded concentrations of SSC, washed in 0.1X SSC at 65°C for 1 hour, and dehydrated in graded EtOH. When dry, slides were laid out in light-tight autoradiography cassettes and exposed to X-ray films (Kodak) for 1 week.

Image Analysis for In Situ Hybridization

Levels of BDNF mRNA were analyzed by computer-assisted optical densitometry. Brain sections images 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 (Scion, Frederick, MD, USA). A macro was written that enabled signal above the background to be determined automatically. For each section, a background sample was taken over an area of white matter, and the signal threshold was calculated as mean gray value of background +3.5 standard deviations. The section was automatically density-sliced at this value, so that only pixels with gray values above these criteria were included in the analysis. Results are expressed as mean integrated density, which reflects both the signal intensity and the number of pixels above the assigned background (mean signal above background×number of pixels above background). Each subject's mean integrated density at a given level represents the average of between 2 and 4 slices chosen for analysis between the following coordinates: Prefrontal cortex from +3.2 to +1.7 mm anterior to bregma; hippocampus and amygdala from -2.3 to -3.3 mm posterior to bregma based on the atlas by Paxinos and Watson (Paxinos and Watson, 2006). Templates for each region were made to ensure that equivalent areas were analyzed between animals.

Data Analysis

Running distances were analyzed using repeated measures ANOVAs. Running distance over the entire 6 weeks was also compiled and compared using ANOVA. . Body weights were similarly analyzed with a 2 x 2 repeated measures ANOVA with Age and Exercise status as between-subjects factors. BDNF mRNA levels in the hippocampus, amygdala and motor cortex were analyzed with 2 X 2 X 2 ANOVA, with age (adult, early life) and exercise status (run, sed) and time point (0,25) as factors. Actual group sizes varied within and between brain regions because of disruptions in tissue integrity incurred during brain removal, slicing, processing, etc. Fisher protected least significant differences (F-PLSD) *post hoc* analysis was conducted only when a significant interaction was observed. Significant effects were detected at a p value of 0.05 or less. Data are represented by means +/- SEM.

Results

In Situ Hybridization Probes

A DNA probe for insulin-like growth factor-1 (IGF-1) was successfully cloned using RNA extraction and isolation, polymerase chain reaction (PCR), ligation and transformation techniques. Successful DNA amplification via PCR was confirmed with gel electrophoresis (figure 2). The bands indicate robust amplification of a PCR product that is ~600 basepairs long, thus supporting successful DNA amplification. Identity of the probe was confirmed via DNA sequencing and the probe is ready for *in situ* hybridization; however, due to time constraints the *in situ* hybridization data are not available for this thesis. The probe for brain derived neurotrophic factor (BDNF) was previously available and a successful *in situ* hybridization was run to examine BDNF mRNA expression.

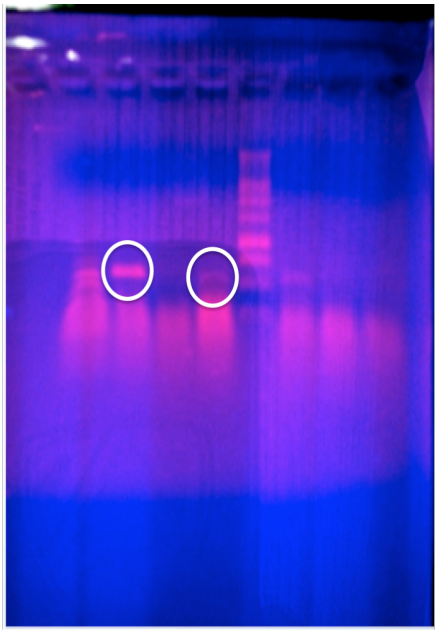

Figure 2

Gel electrophoresis run to confirm DNA amplification via polymerase chain reaction. Gel electrophoresis was run at 100 V for 60 minutes and revealed a bright band around 600 bp.

Running distances and body weight

Average weekly running distance over 6 weeks of voluntary access to a running wheel is shown in Figure 3A. Physically active rats displayed typical running behavior of Fischer 344 rats (Greenwood et al., 2005; Greenwood et al., 2009) increasing distance throughout the first weeks of running followed by a plateau in distance. In general, adult rats increased their running distance through the third week of running and then leveled off at 10.4 ± 1.2 km/week per 2 animals (double housed). ELE animals ran less than AE through the first three weeks of the study but when AE flattened, ELE animals continued to increase running distance, leveling off at week four and maintaining 15.2 ± 0.8 km/ week for the last three weeks of exercise. There was an interaction between time and age of exercise onset ($F(5,110) = 28.9$; $P < 0.0001$, figure 3A). Although ELE animals ran more near the end of the 6 week running period than AE animals, the total running distance over the entire 6 week period did not differ between groups ($F(1,22) = 3.246$; $P=0.0853$, figure 3B).

AE rats gained weight at the same rate as AS rats throughout course of the study. ELE animals, however, gained weight faster than ELS near the end of the running period. Throughout the 6 weeks of exercise there was a main effect of age of exercise onset ($F(1,44) = 3080.94$; $P<0.0001$), an interaction between age of onset and exercise ($F(1,44) = 15.99$; $P=0.002$), a main effect of time ($F(5,220)=548.10$; $P<0.0001$), a time by exercise interaction ($F=(5,220)=3.885$); $P=0.0021$), a time by age interaction ($F(5,220)=45.441$; $P<0.0001$) and a three-way interaction between time, exercise status and age of exercise onset ($F(5,220)=6.351$; $P<0.0001$). Post hocs are shown in figure 3C.

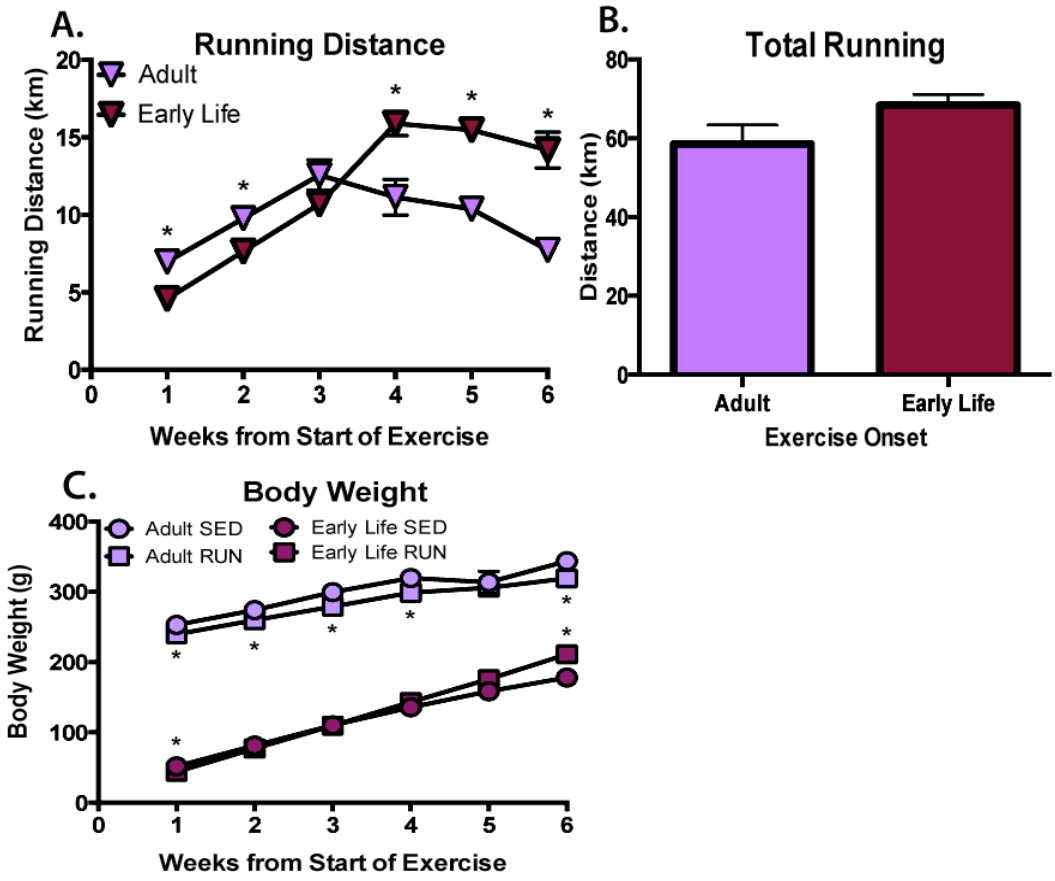

Figure 3.

(A) The mean distance run each week (per 2 animals, pair housed) by male Fischer 344 rats allowed voluntary access to a running wheel for 6 weeks initiated either in early life or adulthood. Post hocs reveal significant differences at week 1 ($P<0.0001$), week 2 ($P=0.001$), week 4 ($P<0.0001$), week 5 ($P<0.0001$) and week 6 ($P<0.0001$). (B) Total distance run over 6 weeks of wheel access. (C) Mean body

weight in grams across 6 weeks of voluntary wheel access or sedentary conditions. Weights between AS and AR groups are statistically different at week 1 (P<0.0001), week 2 (P=0.0001), week 3 (P<0.0001), week 4 (P=0.0002), and week 6 (P=0.0001). The weight between ELS ELE was significantly at week 1 (P=0.008) and week 6 (P<0.0001). Values represent group means ± SEM. * denotes statistically significant, P≤0.05

BDNF in the Dentate Gyrus

ANOVA revealed a significant main effect of time point ($F(1,35) = 5.191$; $P=0.0289$) and a significant main effect of exercise ($F(1,35) = 7.825$; $P=0.0083$). Post-hoc analysis was run because there were 2 main effects. Post hocs revealed that exercise increased BDNF mRNA immediately following the cessation of exercise independent of exercise onset age, but this increase was present at the 25D time point only if in ELE animals (results of post hocs are shown in figure 5). The region quantified for the dentate gyrus is shown in figure 4A. Representative slices for the different groups can be seen in figure 6.

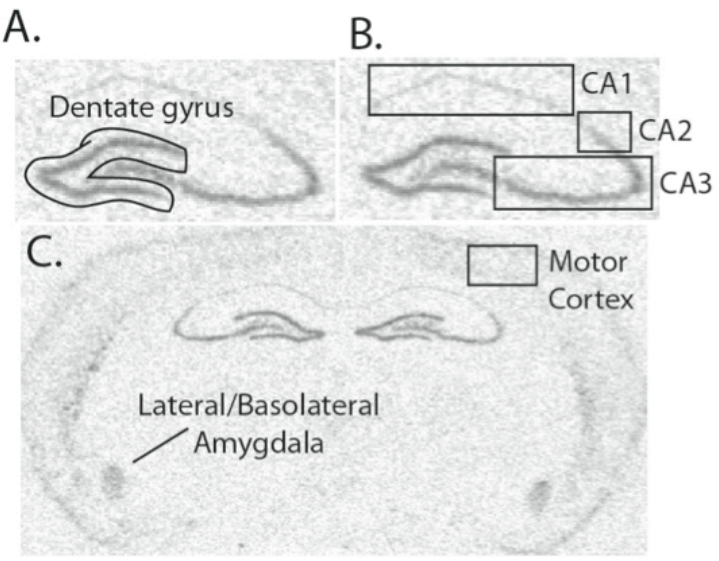

Figure 4

Example of regions quantified. Multiple regions of the hippocampus (dentate gyrus, CA1, CA2, CA3) were quantified as well as the motor cortex and lateral/basolateral amygdala.

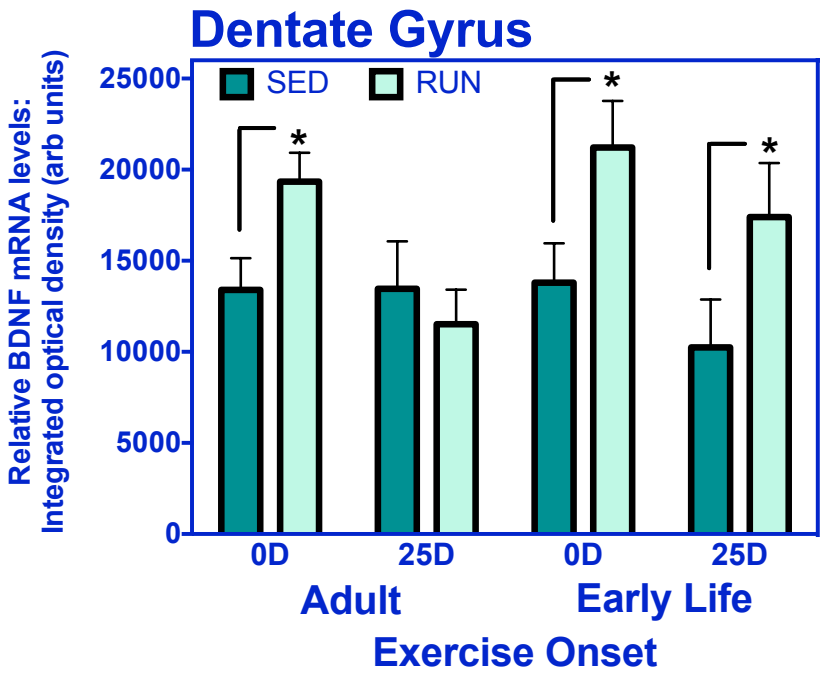

Figure 5

BDNF in the dentate gyrus either immediately (0D) or 25 days (25D) following the cessation of exercise. Post hocs reveal significantly elevated BDNF at the 0D time point regardless of whether exercise was initiated during adulthood (AS) or during early life (ELE). 25 days following the cessation of exercise the adult BDNF mRNA levels are back to basal levels but if running was initiated during early life (ELE) BDNF mRNA is still significantly higher than controls. * P≤0.05

Figure 6

(A-F) Representative autoriadiographic coronal sections through the hippocampus showing relative levels of brain derived neurotrophic factor (BDNF) mRNA in the dentate gyrus (DG), CA3, CA2, and CA1 regions of the hippocampus, motor cortex, and amygdala between groups.

BDNF mRNA decreases across time in other regions of the hippocampus

Exercise did not have a significant effect on BDNF mRNA in the CA1, CA2, or CA3 regions of the hippocampus. There was, however, a main effect of time point in all three regions: CA1 ($F(1,38) = 7.585$;

P=0.0090), CA2 (F (1,38) = 4.722; P=0.0361), CA3 (F (1,39) = 11.828; P=0.0014). Figure 7A-C shows the mean levels of BDNF mRNA within CA3, CA2, CA1, regions of the hippocampus, respectively.

Main effect of time point on BDNF mRNA in the lateral/basolateral amygdala

Figure 7D shows the mean BDNF mRNA levels in the lateral/basolateral amygdala. There was a significant effect of age of exercise onset (F (1, 38) = 7.454; P=0.0095) as well as an interaction between age of exercise onset and time point (F (1,38) = 6.081; P=0.018). The youngest animals had significantly elevated BDNF levels in the lateral/basolateral amygdala compared to all other groups, independent of physical activity status (figure 7D).

No effects of age or running in the motor cortex

Physical exercise and age did not have any significant impacts on BDNF mRNA in the motor cortex. Figure 7E shows the mean BDNF mRNA in the motor cortex.

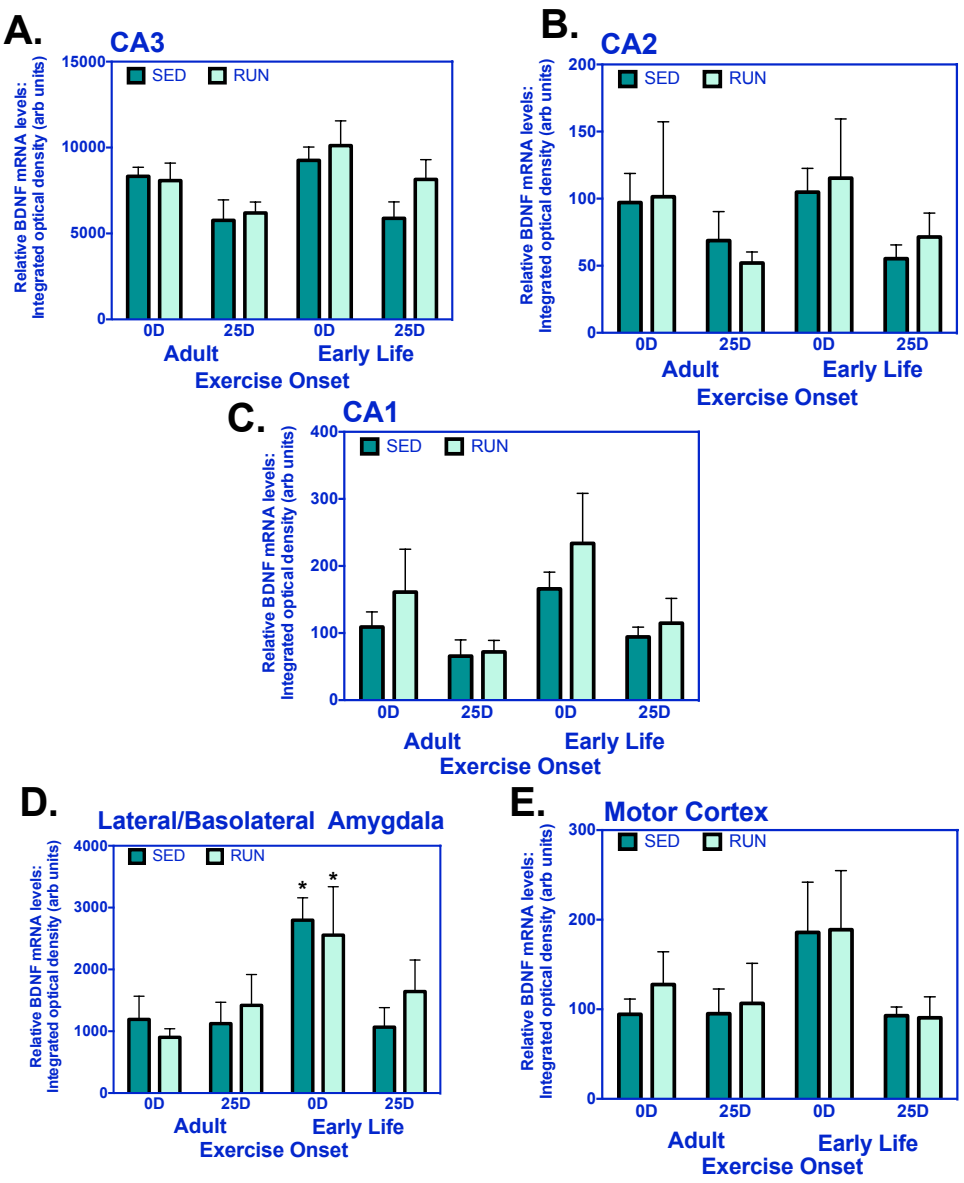

Figure 7

There is no effect of exercise on BDNF mRNA expression in other regions of the hippocampus, lateral/basolateral amygdala or cortex. (D) Post hoc analysis of the lateral/basolateral amygdala reveals significantly increased BDNF mRNA levels 0D time point of the early-life exercise animals, independent of exercise status, as compared to all other groups. * $P \leq 0.05$

Discussion

While the ability of exercise to increase neural growth factor mRNA is well established in adults, the effect of exercise during development is less clear. In this study, rats initiated 6 weeks of voluntary

wheel running either in early life (ELE) or adulthood (AE) after which mRNA expression was examined via *in situ* hybridization immediately and 25 days following the cessation of exercise. Exercise animals were compared to either early life (ELS) or adult (AS) sedentary controls. We hypothesized that the well-established increase in growth factor mRNA immediately following exercise would be more persistent if exercise was initiated during early life. This hypothesis was confirmed!

Consistent with the literature, exercise significantly increased adult BDNF mRNA in the dentate gyrus region of the hippocampus (Greenwood et al., 2007; Greenwood et al., 2009; Oliff et al., 1998). Our results vary slightly from Dr. Greenwood's 2009 publication, which reported an increase in the CA1 region and basolateral amygdala (BLA) as well as the dentate gyrus; however, these increases were seen only in the mid- and caudal-CA1 and the mid-BLA in adolescent (P~49) rats (Greenwood et al., 2009). Given that our study focused on the rostral hippocampus and lateral/BLA and our age of exercise onset was different, it is not surprising that we did not observe increases in these other regions. In addition to this expansion of previously published data within different age groups, the current data demonstrate that the BDNF mRNA increase is more persistent if running is initiated during early life (ELE), compared to adulthood (AE). This difference is clearly illustrated graphically in figure 5 and can be seen visually in figure 4. Immediately following exercise, BDNF mRNA is elevated equally between the ELE and AE; however, 25 days following the cessation of exercise ELE animals continue to have increased BDNF mRNA levels while AE animals are back to baseline.

Interestingly, other regions of the hippocampus (figure 6) showed a main effect of time not dependent on exercise status. The time point effect observed in these regions is reflective of the age of the animals at the given time points of sacrifice rather than the time following physical activity cessation. Indeed, the ELE/ELS 25D animals and the AE/AS 0D animals are close in age (20 days apart). These data

therefore suggest that brain BDNF mRNA levels are highest during early life and subsequently decrease with age, as has been previously reported in the literature (Karege et al., 2002).

Although ELE and AE animals ran the same distance throughout the entire running regimen (figure 3B), their running pattern was different (figure 3A). This is a potential confound to the study, as our data could simply reflect a dose response. Even so, our data show that if allowed voluntary access to running wheels early in life, exercise-induced BDNF mRNA changes are more persistent. The animals were allowed to run as much or as little as they wanted and the young animals chose to run more. The voluntary participation in higher amounts of exercise during early life may be an important aspect of the persistent effects; this choice may be beneficial in that it may promote longer-lasting effects of exercise.

The persistent effects of exercise-induced BDNF levels in ELE animals mirror the persistent behavioral effects of ELE. ELE produces long-lasting improved performance in non-spatial memory tasks such as novel object recognition (Hopkins et al., 2011) as well as spatial learning tasks like the Morris water maze (Gomes da Silva et al., 2012). While the former is not hippocampal dependent and the latter is dependent upon the hippocampus, they are both likely dependent upon BDNF. BDNF initiates intracellular cascades that are critical for formation and consolidation of a memory (Cunha et al., 2010). Long-lasting anxiolytic and antidepressive effects of exercise have also been shown following early life exercise (Mika et al., unpublished observations). Infusion of BDNF into the dentate gyrus produces anti-depressant effects in models of depression (Shirayama et al., 2002) and defective BDNF signaling leads to higher anxiety when exposed to stressful situations (Chen et al., 2006). Given the importance of BDNF in these cognitive functions and the persistence of exercise-induced BDNF within the dentate gyrus found in this study, increased BDNF levels could be behind the documented persistence of behaviors following ELE.

IGF-1 has the potential to interact with BDNF to mediate the interaction between the critical period of development and the exercise. An IGF-1 probe was successfully generated using molecular techniques and is ready for *in situ* hybridization; however, due to time constraints the *in situ* data are not available for this thesis. Even so, IGF-1 expression is highly associative with exercise and may play a pivotal role in the persistence of exercise-induced benefits following early life exercise. Evidence shows that transgenic mice over expressing IGF-1 postnatally in the brain leads to a persistent elevation in total number of neurons and synapses within the dentate gyrus (O'Kusky et al., 2000). Interestingly, IGF-1 modulates BDNF activity levels. Landi et al. (2009) reported that IGF-1 injection into the retina of developing (non-enriched) rats increases the BDNF expression while injecting an IGF-1 antagonist blocks the enhanced BDNF expression generally found after environmental enrichment (Landi et al., 2009). Others have reported that IGF-1 signaling mimics the exercise-induced increase in c-fos and BDNF mRNA in the hippocampus (Carro et al., 2000). IGF-1 is a potential factor involved in effects of antidepressant medications as increased levels of IGF-1 leads to anxiolytic-like effects (Malberg et al., 2007). Given the importance of IGF-1 signaling in anxiolytic, depressive and learning and memory tasks and also the importance of IGF-1 signaling for increases in BDNF expression, further investigation into IGF-1 will help to form a more complete story of early life exercise on persistent cognitive functions.

The ELE animals in this experiment exercised through a very important developmental period. During development, there are two major waves of synaptic pruning, one is right before birth and the other one is around puberty (for review see (Rice and Barone, 2000). These pruning periods are classified by immense apoptosis of unnecessary neurons, pruning is a way for the brain to compact its necessities and rid the unnecessary connections and neurons. Interestingly, a hallmark of both BDNF and IGF-1 is the activation of anti-apoptotic cascades; thus, neurons being acted upon by BDNF or IGF-1 during a period of immense synaptic pruning will not be “pruned away”. In this study, we manipulated

the developmental period before and during puberty. The ELE animals began running during early life (P24) and the running paradigm continued through puberty. This behavioral manipulation increased BDNF in the dentate gyrus, which perhaps may protect these specific neurons from the apoptosis that occurs in the second wave of synaptic pruning. This modification to the normal maturation of the central nervous system may lead to profound changes in adult brain function.

This study revealed a persistent increase in a well-documented exercise-induced neural change. Importantly, future quantification of *IGF-1* mRNA within the same regions as BDNF from this study will further the mechanistic details of the persistence of the exercise-induced effects and help to determine the importance of growth factors during a critical period of development. The persistence of exercise-induced BDNF mRNA following ELE revealed by this study has important implications and may contribute to the persistence of the exercise effect following ELE. It is well documented that exercise has therapeutic benefits and finding the mechanism behind the powerful, beneficial effects of exercise will better enable us to harness these benefits.

Conclusion

Radioactive *in situ* hybridization revealed an increase in BDNF mRNA in the dentate gyrus region of the hippocampus following 6 weeks of voluntary wheel running. This increase was significant in both ELE and AE; however, 25 days following the cessation of exercise, AE BDNF mRNA levels had returned to baseline while ELE showed a persistent effect. This persistence mirrors well-documented persistence in exercise-induced behaviors such as anxiolytic and antidepressant effects as well as learning and memory enhancements following ELE. In order to form a more complete story, an IGF-1 probe was cloned and prepared for *in situ* hybridization. This thesis begins to unveil a possible mechanism behind the persistence of exercise-induced effects of exercise initiated in early life.

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