

**EFFECTS OF VOLUNTARY RUNNING ON BRAIN
MITOCHONDRIAL BIOGENESIS IN RATS
SELECTIVELY BRED TO RUN HIGH AND LOW
NIGHTLY DISTANCES.**

A Thesis

**Presented to the Faculty of the Graduate School
at the University of Missouri**

In Partial Fulfillment of the Requirements for the Degree:

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by

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

EFFECTS OF VOLUNTARY RUNNING ON BRAIN MITOCHONDRIAL
BIOGENESIS IN RATS SELECTIVELY BRED TO RUN HIGH AND LOW
NIGHTLY DISTANCES.

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and hereby certify that, in their opinion, it is worthy of acceptance.

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Effects of Voluntary Running on Brain Mitochondrial Biogenesis in Rats Selectively Bred to Run High and Low Nightly Distances.

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ABSTRACT

It is known that brain mitochondrial dysfunction is strongly associated with the advent of neurodegenerative diseases and that exercise is an important non-pharmaceutical method of maintaining brain mitochondrial health. Many models have been used to examine the mechanisms by which exercise increases the concentration of brain mitochondria, but it is unknown whether certain genetic factors associated with the motivation to exercise play a role in the biogenesis of mitochondria in different segments of the brain as a result of voluntary running. Therefore, the purpose of this study was to determine whether innate differences in the motivation to voluntarily run play a role in mitochondrial biogenesis of the nucleus accumbens (NAc), ventral hippocampus (HC), and the lateral hypothalamus (LH) as a result of exercise. **Methods:** Dr. Booth has previously reported a novel model in which Wistar rats were selected over several generations to produce two novel lines with the characteristics of high motivation to voluntarily run (HVR) and low motivation to voluntarily run (LVR). Building upon this model, HVR, LVR, and wild type (WT) rats were randomly selected at 28-34 days of age and divided into two groups: those that were given a voluntary-running wheel (RUN) and those without a voluntary-running wheel (SED). For a period of 5 weeks, voluntary-wheel running distance, and duration

was recorded daily, while food intake and body weight were recorded each week.

Western blotting was performed on the NAc, HC, and LH brain tissue to examine PGC-1 α and mitochondrial respiratory chain complexes 1-5. **Results:** Despite the inherent differences in motivation to run between the selectively bred rat lines and the WT rats, data suggests that the LVR SED rats possessed the highest content of PGC-1 α as well as mitochondrial respiratory chain complexes. **Conclusions:** These results suggest that, perhaps, the selective breeding model unintentionally co-selects for genes resulting in the LVR SED animals having a higher content of mitochondria in the selected portions of brain tissue. It is also possible that the SED animals, being dual-housed, conferred this advantage as a result of cage activity and social stimulation rather than voluntary running.

INTRODUCTION

Introductory paragraph

The health significance of my study is that 1) loss of cognitive function with aging is associated with lower brain mitochondrial function; and 2) physical activity increases brain mitochondria in young (Kirchner et al., 2008) and old (E, Burns, & Swerdlow, 2014) mice, thus countering mitochondrial dysfunction. The World Health Organization (WHO) projects that as soon as 2040, neurodegenerative diseases, such as Alzheimer's and Parkinson's disease will surpass cancer to become the second leading cause of death, following cardiovascular disease. Furthermore, diseases of cognitive function lower quality of life for a very prolonged duration at the end of life. Low mitochondrial density in the brain is associated with an increased risk for development of neurodegenerative disease (Lezi & Swerdlow, 2012). In contrast, an increase in mitochondrial biogenesis in the brain is associated with a prevention of neurodegenerative conditions, as well as a reduction in their clinical consequences once they are present. There is evidence that Alzheimer's disease (AD) has a genetic inheritance. Family history is the second leading factor associated with the occurrence of AD after aging (Tanzi, 2012). AD results from a combination of genetic and environmental interactions. Aerobic exercise has been well documented to promote the biogenesis of mitochondria in many brain tissues. For example, hippocampus and hypothalamus tissues have been found to undergo mitochondrial biogenesis in response to exercise training (Bayod et al., 2015; Steiner, Murphy, McClellan, Carmichael, & Davis, 2011). Although, inherited fitness factors like

motivation for exercise have been strongly associated with genes, there is evidence to show that cardiovascular fitness is not entirely inherited. In laboratory animals, environmental factors, such as, the presence of a wheel to allow for voluntary running have been associated with increased brain mitochondria (Boveris & Navarro, 2008).

Epidemiology of Brain Diseases

A role has been shown for lack of physical activity in brain dysfunctions. Importantly, physical activity levels have been decreasing in the U.S., accelerating the urgency to understand the biological basis of gene-environment (physical inactivity) interaction to identify mechanisms for therapy. Neurodegenerative diseases affect millions of individuals each year in the United States. The Alzheimer's Association estimates that 5.4 million individuals currently have Alzheimer's disease and in the next 35 years that amount will double (Association, 2015). Currently, 50,000 new cases of Parkinson's disease are diagnosed each year according to the US National Institute for Neurological Disorders and Stroke (NINDS). In the face of a looming mental health crisis, research has determined that physical activity is an effective method of prevention of neurodegenerative disorders as well as mitigation of symptoms in those already affected. Large population studies of young to middle-aged adults suggest that higher levels of moderate to vigorous physical activity are associated with reduced risk of Parkinson's disease (PD) (Xu et al., 2010). Similarly, observational studies have implicated high levels of activity amongst a middle-aged populations with reducing the incidence of dementia (Defina et al., 2013). In addition, physical activity has also been found as a successful treatment in reducing symptoms of patients with Huntington's disease (Khalil et al., 2013; Spires et al., 2004). As illustrated by the above examples, physical activity is

an effective, non-pharmaceutical means of prevention and treatment of many neurodegenerative diseases.

Exercise Epidemiology

Sedentary lifestyle or “couch potato” lifestyle is a major risk factor for obesity.

Accelerometer data suggests that 58% of children 6-11 years of age and 92% of adults in the United States do not meet the recommended 60 minutes of physical activity each day (Troiano et al., 2008). With the increased incidence of the “couch potato” lifestyle amongst Americans, the magnitude of the obesity problem does not come as a surprise. According to the National Health and Nutrition Examination Survey, conducted from 2009-2010, more than 66% of adults in the United States are considered to be overweight or obese according to standard Body Mass Index (BMI) parameters. The same study also found that as many as 33% of American children, between the ages of 6-19 years of age, are considered to be overweight or obese. Obesity has been associated with an increased risk for developing coronary heart disease, high blood pressure, stroke, type 2 diabetes as well as many other diseases, according to the National Heart, Lung, and Blood Institute. Annually, obesity-related illness in adults accounts for more than 20.6% of U.S. health expenditures and costs the United States \$209.7 billion in 2008 dollars (Cawley & Meyerhoefer, 2012).

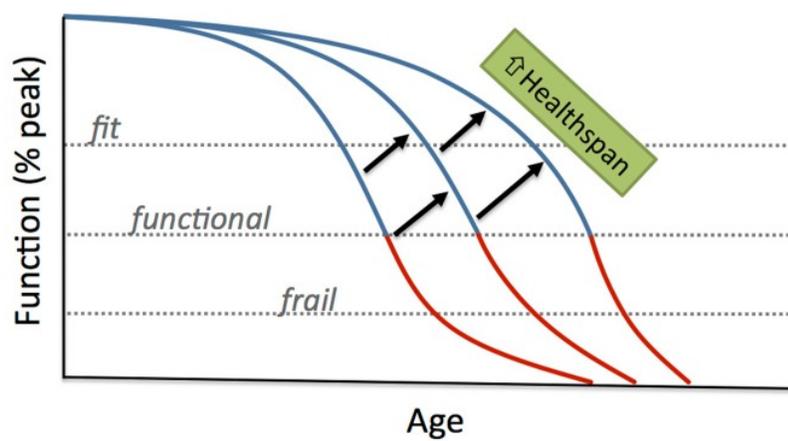
Integrating the Previous Paragraphs

Two notable trends have developed in modern society that have drastic effects on healthcare and lifestyle. As a result of modern medicine, longevity has increased in humans substantially ("Global health and ageing," 2011). In many neurodegenerative

diseases, aging is among the greatest of risk factors. For example, in the most common form of dementia, AD, senescence is considered to be one of the greatest risk factors (Antuono & Beyer, 1999; Imtiaz, Tolppanen, Kivipelto, & Soininen, 2014). Also as a result of modern society's production of effort-sparing technology, there is less of a need for physical activity in daily lives, which has contributed to the predominance of a sedentary lifestyle. One decade ago, a potential decline in lifespan was prophesied by Olshansky et al. (Olshansky et al., 2005). Implicit in shorter lifespans is a shorter healthspan. Healthspan can be defined as the period of a person's life during which they are free from chronic diseases (Kirkland & Peterson, 2009). Although life-long exercise does not increase longevity, it has been established as an effective method of treatment to increase healthspan, which results in an increased period of independence without the advent of disease or frailty (Garcia-Valles et al., 2013). In Western societies longevity exceeds healthspan by a number of years resulting in many years being spent living with debilitating disease. Thus, it is likely that neurodegenerative diseases will continue to increase in prevalence as sedentary lifestyles become more common.

Figure 1. From Seals et al. 2014, “Compression of Dysfunction” with Aging

Successful treatments, such as exercise, can be utilized in order to limit the loss of function that occurs from chronic disease to the end of life.



Mitochondrial Energetics Basis

Mitochondrial bioenergetics play a central role in basic cellular structure and function and are interrelated to environmental factors, such as diet and exercise. The mitochondria are the main providers of cellular energy, which is created through the process of oxidative phosphorylation (OXPHOS) in the form of ATP.

Oxidative phosphorylation and the Electron Transport Chain (ETC)

OXPHOS is the process by which the cell produces energy by utilizing the mitochondrion to oxidize the reducing equivalents, NADH and flavin adenine dinucleotide (FADH₂), with oxygen to form water and generate cellular energy in the form of adenosine triphosphate (ATP) (Wallace, Fan, & Procaccio, 2010). The remainder of the paragraph is summarized from Wallace et al. 2009. In order for ATP production to occur, glucose must be cleaved to pyruvate by phosphofructokinase-1 (PFK-1) in the cytosol, which reduces cytosolic nicotinamide adenine dinucleotide (NAD) to NADH. Pyruvate dehydrogenase (PDH) cleaves pyruvate into acetyl coAs, which generates NADH + H⁺ and allows it to enter the mitochondrion. This mitochondrial acetyl coA then enters the tricarboxylic acid (TCA) cycle, which utilizes the H⁺s to generate additional NADH + H. β-oxidation inside the mitochondrion oxidizes fatty acids in order to generate acetyl coA, NADH + H⁺ and FADH₂. The electron transport chain (ETC) begins when the reducing equivalents, NADH + H⁺ and FADH₂ transfer their electrons through oxidation to NADH dehydrogenase (Complex I) and succinate dehydrogenase (Complex II) respectively. NADH from glycolysis, the TCA cycle, and β-oxidation are reoxidized to NAD⁺, while FADH₂ from the TCA cycle is oxidized to FADH. This transfer of electrons by oxidation serves to link the ETC with the other metabolic processes of producing energy. These electrons are then transferred

further to ubiquinone (CoQ), which is in turn reduced to ubiquinol in a process known as the “Q” cycle. Once reduced to ubiquinol, the electrons are sequentially transferred down to the subsequent portions of the ETC to cytochrome c oxidoreductase (Complex III), to cytochrome c oxidase (Complex IV (COX)) through cytochrome c, and then to the final electron receptor, oxygen, which yields H₂O. Complexes I, III, and IV create a proton electrochemical gradient causing the mitochondrial matrix to be negative and the outside of the inner mitochondrial membrane to be positive by pumping protons out of the inner mitochondrial membrane as the electrons are transferred through the ETC. The energy potential from the proton flux of this gradient is used by ATP synthase (Complex V) to convert adenosine diphosphate (ADP) + inorganic phosphate (Pi) to ATP for use as cellular energy. The efficiency of this process is referred to as the coupling efficiency. Physiological stimuli can cause changes in mitochondrial proteins that can affect the efficiency by which protons are pumped out of the matrix, which results in a reduced efficiency of complex V’s production of ATP.

Function of the Mitochondrion in Healthy Organisms and Role in Regulation of Apoptosis

The core functions of mitochondria in the cell are the production of ATP to be used as cellular energy, the regulation of reactive oxygen species (ROS), control of cytosolic calcium homeostasis, and the regulation of programmed cell death through the mitochondrial permeability transition pore (mtPTP). The adult human brain almost exclusively depends on glucose metabolism for energy and has nearly no oxygen stores, mitochondrial PO₂ concentration being only a few mmHg PO₂. Cells with defective mitochondria utilize the mtPTP to initiate programmed cell death. Mitochondrial stress

can occur as a result of free radicals and oxidative damage. When excess mitochondrial stress occurs, large amounts of Ca^{2+} are taken up by the mitochondria, which results in the opening of the inner mitochondrial membrane, which short-circuits the energy generating proton gradient of the ETC and initiates cellular degeneration and apoptosis. The mtPTP is also regulated by mitochondrial oxidation status, which as a result of high levels of ROS can lead to apoptosis. The correlation between oxidative stress and apoptosis is closely related to the pathophysiology of many neurodegenerative diseases including AD and PD (Calabrese, Scapagnini, Giuffrida Stella, Bates, & Clark, 2001).

Impaired Mitochondrial Function and Neurodegenerative Disorders

Many neurodegenerative diseases share the phenotype of impaired mitochondrial function (Friedland-Leuner, Stockburger, Denzer, Eckert, & Muller, 2014; Moon & Paek, 2015; Okazawa, Ikawa, Tsujikawa, Kiyono, & Yoneda, 2014). Commonly, these dysfunctions manifest through increased production of reactive oxygen species (ROS) and their corresponding oxidative damage and decreases in the oxidative phosphorylation capacity of the respiratory chain.

Peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1 α), is a prominent controller of oxidative metabolism. PGC-1 α has been demonstrated to ameliorate many of the major causes of mitochondrial dysfunction through its actions as a master regulator of mitochondrial biogenesis and its ability to alter the intrinsic properties of the mitochondria organelle (Sheng et al., 2012; Zheng et al., 2010). Although, an increase in mitochondria increases the amount of ROS output into the cell through mitochondrial metabolism of the respiratory chain complexes, PGC-1 α , also increases the detoxification

of ROS by up-regulating ROS detoxifying enzymes (Austin & St-Pierre, 2012; St-Pierre et al., 2006).

The exact machinery involved in the cross-talk between exercising muscle and non-muscle tissues, such as the brain, is not well understood. Recently experiments by Swerdlow et al., implicate lactate as playing at least a partial role in the bioenergetics adaptations associated with exercise training that occur in the brain (E, Lu, Selfridge, Burns, & Swerdlow, 2013). It is likely that adaptations are region specific and occur based on specific brain-tissue's bioenergetics needs.

It has been demonstrated that a lack of PGC-1 α in the brain is associated with impaired mitochondrial function and neurodegeneration (J. Lin et al., 2004). There are numerous animal models that have demonstrated the protective abilities of PGC-1 α against neurodegenerative disease. Furthermore, studies have implicated that exercise increases mitochondrial biogenesis in several portions of the brain through a PGC-1 α -mediated mechanisms (Steiner et al., 2011).

A Model of High and Low Physical Activity

Over the course of the last 6 years, our lab has developed two novel lines of Wistar rats selectively bred for high- and low-voluntary running, HVR and LVR, respectively. In the founding population, at 28 days of age rats were introduced to a running wheel (circumference: 0.345 m) and ran while being monitored by Sigma Sport BC 800 bicycle computers (Cherry Creek Cyclery, Foster Falls, VA) for a period of 6 days. The lines were then selected based upon which rats ran the highest (HVR) or lowest (LVR) average running distance on nights 5 and 6 and paired for mating in a 13 family scheme to

minimize inbreeding. Over the following generations, the two phenotypes became characterized by a large gap in running differences, as well as differences in skeletal muscle, body composition, and transcriptomic differences in genetic drives for motivation attributed to the nucleus accumbens (NAc) (Roberts et al., 2013). The NAc is considered the major reward and pleasure hub of the brain and has been attributed as a regulator of voluntary physical activity (Knab & Lightfoot, 2010; Rhodes, Garland, & Gammie, 2003). A study conducted by Roberts et al., (Roberts et al., 2014) suggested that there are gene expression differences between the lines that resulted in decreased maturation of NAc neurons in LVR rats in comparison to HVR, which may be contributing to their decreased motivation to voluntarily run. Interestingly, when LVR rats are provided a wheel they are able to increase the amount of mature NAc neurons, which suggests that environmental factors also play a role in motivation to exercise (Roberts et al., 2014).

High densities of mitochondria in the brain are strongly correlated with good metabolic health as well as prevention of neurodegenerative diseases such as, Alzheimer's and Parkinson's (Flynn & Melov, 2013). To the best of my knowledge, the measurement of mitochondria in the nucleus accumbens, an area of the brain associated with motivation for exercise, will be novel. A higher density of mitochondria promotes exercise-associated health benefits such as attenuation of senescence-associated cognitive declines, decreases in mental fatigue, and improved performance (Bayod et al., 2015; Steiner et al., 2011). It is well known that a sedentary lifestyle is associated with a decline in mitochondrial density, which has been linked to the advent of numerous diseases and neurodegenerative disorders (Dunham-Snary & Ballinger, 2013; Stephenson

& Hawley, 2014; Sun, Zhang, & Liu, 2014). However, controversy exists as to whether specific genes contribute to the amount of aerobic exercise required for individuals to stimulate mitochondrial biogenesis in their brain tissue.

Thesis Objective

The purpose of this experiment was to determine whether voluntary wheel running would produce equivalent increases in mitochondrial biogenesis between subjects genetically predisposed to sedentary lifestyles when compared to subjects genetically predisposed to active lifestyles utilizing the Booth low motivation voluntary running (LVR) and high motivation voluntary running (HVR) rat models, respectively. I hypothesized that the selected brain tissues of rats remaining sedentary would present fewer increases in markers of mitochondrial biogenesis, such as the master regulator, PGC-1 α , and yield fewer increases in markers of mitochondrial OXPHOS; whereas, rats with increased motivation for high voluntary running activity through would yield a higher density of mitochondria. This is based upon the fact that aerobic exercise increases the oxidative stress on both skeletal muscle tissue and brain tissue, which stimulates the biogenesis of mitochondria in these tissues (Garcia-Mesa et al., 2015; Mattson, 2015; Steinbacher & Eckl, 2015; Steiner et al., 2011; Wang, Li, Qi, Cui, & Ding, 2015). The current investigation utilized two novel lines of Wistar rats selectively bred for high- and low-nightly voluntary wheel running distances along with wild type (WT) rats. The differences in motivation to run are representative of the differences in athletes predisposed to high levels of physical activity (the HVR line) and those who prefer to live a “couch potato” lifestyle (the LVR line) (Roberts et al., 2013). To test for physical activity influences imposed upon the two intrinsic genetic models, approximately half of

each group was given a running wheel while their counterparts remained sedentary. In addition, I included a wild-type group for further genetic comparison. The advantage of the experimental design is that the presence of the highly motivated voluntary runner group (RUN) and the group with low motivation for voluntary running (SED) allows for analysis of not only genetic differences in motivation, but also the interactions of the genes with high and low running duration of a voluntary-running wheel. The aim of my research was to determine gene: physical activity interactions on mitochondrial protein values in specific areas of the brain. I hypothesized that the RUN rats, after 5 weeks of wheel running will have a significantly higher levels of PGC-1 α and OXPHOS proteins than their SED counterparts. I also expect that the HVR RUN group, as a result of larger volumes of running, will have the largest increase in these proteins due to physiological adaptation to exercise. I further hypothesize that the LVR RUN group will have a significantly lower increase in mitochondrial proteins as a result of a lower level of voluntary running.

MATERIALS AND METHODS

Animals and Selective Breeding

The Institutional Animal Care and Use Committee at the University of Missouri-Columbia approved all animal experiments. LVR, HVR, and WT rats were bred at the University of Missouri. Rats were housed in temperature-controlled facilities on a 12 hour: 12 hour light: dark cycle and allowed *ab libitum* access to food and water. The Booth laboratory has selectively bred Wistar rats based upon high voluntary running (HVR) and low voluntary running (LVR) differences through multiple generations. This has produced an extremely large difference between the amounts of voluntary running activities between the two groups. This experimental design is outlined in (Roberts et al., 2013).

Experimental Design

28 to 34-day-old HVR, LVR, and wild type (WT) female, Wistar rats will be randomly separated into two groups. One group was the voluntary running group, which is composed of 36 rats who will be provided with a voluntary running wheel and the other, the sedentary group, was composed of 29 rats without a running wheel. Daily measurements of nightly running distance and duration were recorded. Measurements of body weight and food consumption were recorded on a weekly basis. At the end of the 5-week treatment period the rats were sacrificed and the nucleus accumbens (NAc), ventral hippocampus (HC), and lateral hypothalamus (LH) were extracted from the brain. Skeletal muscle tissue was also extracted and weighed from the soleus, gastrocnemius,

and the plantaris muscles. Tissues were analyzed for proteins associated with mitochondrial dynamics by Western blotting assay.

Voluntary Wheel Running

We analyzed the effects of 5 weeks of voluntary wheel-running in 28-34-day-old selectively bred, LVR and HVR rats as well as WT rats. After the 5-week treatment period, the rats were approximately 70 days old. The running wheels (circumference: 0.345 m) were monitored utilizing Sigma Sport BC 800 bicycle computers (Cherry Creek Cyclery, Foster Falls, VA).

Food Intake and Body Weights

Measurements of total mass of standard chow (Formulab Diet 5008) added, total mass of food remaining, and body weight were recorded of each test subject weekly.

Extraction of Tissue

At the time of sacrifice, tissues extracted were the NAc, HC, LH, soleus, plantaris, and gastrocnemius. Both brain tissue and skeletal muscle tissue were frozen instantaneously in liquid nitrogen after extraction. Brain tissue was extracted using a 3-mm hole-punch (Braintree Scientific, Brain-tree, MA, USA). Tissue plugs from brain regions were verified as their intended region visibly (Watson, 1997). A 3-mm hole punch was used to extract the NAc, HC, and LH. Skeletal muscle (red gastrocnemius, plantaris, and soleus) were extracted. All tissues were immediately flash frozen in liquid nitrogen, and stored at -80°C.

Weighing of Muscle Tissue

Measurements of the mass of the soleus, plantaris, and gastrocnemius were recorded at the time of sacrifice and tissue extraction on an analytical balance scale (Mettler Toledo). Analysis included skeletal muscle weights to total body weight at the time of sacrifice.

Tissue Processing and Immunoblotting

Equivalent protein amounts of NAc, HC, LH, and red gastrocnemius tissue from 65 Wistar rats was homogenized using steel ball homogenizers (2X 20Hz for 1 min) in 400 ul of Rippa buffer containing 69.44 mM Tris Base (pH 8.0), 1.5 M NaCl, 10% NP-40, 10% Na deoxycholate, 10% SDS, as well as phosphatase inhibitors II, III, and complete protease inhibitor II. Tissue samples were placed in 2ml bead homogenization tubes using two steel beads and homogenized in a TissueLyser LT (Qiagen) with two repetitions at 20 Hz for 1 minute. The homogenate was gently rotated at 4°C for 30 min in order to allow the action of both phosphatase and protease inhibitors. This was followed by centrifugation at 4°C for 10 min (12,000g). The protein concentration was determined using BCS Protein Assay (Thermo Scientific) with bovine serum albumin (BSA) dilutions used for a standard curve. Protein content was then quantified spectrophotometrically at 562 nm (SAFIRE, XFLOUR 4) in order to determine the concentration of samples for Western blot preparation. Homogenate was prepared for gel electrophoresis using premade 4X buffer (125mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 5% β -mercaptoethanol, and 0.01% bromphenol blue), which had been frozen at -20°C. Brain and skeletal muscle homogenates in loading buffer were separated electrophoretically on 18-well 4-15% Criterion TGX Precast Gels (Bio-Rad) using a (Precision Plus Protein All Blue Standards #161-0373) and (BLUEstain™ GOLDBIO.com #1007-508) protein ladders at 200V for a period of 45 minutes. Proteins

were then transferred onto Immun-Blot PVDF Membrane for Protein Blotting (Bio-Rad) at 100V for a period of 1 hour. Following protein transfer, membranes were blocked in 5% NFDM-TBS solution containing 2% dry milk for 1 hour at room temperature then incubated with primary antibodies for Anti-PGC-1 α (Millipore and SantaCruz) at 1:1000 dilution, and Anti-Rat/Mouse Total OxPhos Complex Kit (Invitrogen Cat#458099) at 1:10000 and 1:5000 dilutions. Primary antibodies were diluted in 5% NFDM-TBS solution overnight at 4°C. The following day, membranes were vigorously washed with 1X TBS-T for 4 repetitions of 5 minutes on an orbital shaker (Gene Mate). After adequate washing, membranes were incubated for 1 hour at room temperature with (1:1000 dilution) anti-rabbit IgG, HRP-linked antibody (Cell Signaling) and (1:2000) anti-mouse IgG, HRP-linked antibody (Cell Signaling). Band densitometry was accomplished using a Kodak 4000R Imager and Molecular Imagery Software (Kodak Molecular Imaging Systems, New Haven, CT, USA).

Statistical Analysis

Data analysis is expressed as mean \pm standard error of the mean (SEM). Statistical analysis was carried out using Sigma Plot software. Two-way (line type (LVR vs. HVR vs. WT) \times activity (RUN vs. SED) analysis of variance (ANOVA) was utilized in order to assess the main effects of the selectively bred line and activity treatment. Multiple comparison procedural analysis were carried utilizing the Holm-Sidak method. However, in several instances, the Student-Newman-Keuls method was used when the Holm-Sidak was not able to isolate which group(s) differed from the others to better elucidate the observed data. Use of the Student-Newman-Keuls was not necessary to accurately observe the difference between groups in most cases. Analysis and comparison between

the lines and activity were achieved through use of the two-tailed Student's *t* test for independent groupings of samples. Statistical significance was set at $p < 0.05$ for all comparisons.

RESULTS

Running Data:

Measurements of voluntary-wheel running distance were recorded daily. After 35 days of voluntary running there was a statistically significant difference between all lines (HVR>WT>LVR) ($P<0.001$) (**Fig. 2 a**). At 35 days of running LVR RUNs ran an average cumulative running distance of 66.3 Km. As indicated by their assigned nomenclature, the LVR RUNs ran the lowest distance after 35 days. Next in order of distance were the WT RUNs, who ran an average cumulative running distance of 306.44 Km over the course of the 35 day study. Interestingly, the WT RUNs fell nearly perfectly in between their selectively bred counterparts. Further, as indicated by their assigned nomenclature, the HVR RUNs ran an average cumulative running distance of 600.97 Km, which was found to be the highest distance. Daily running time was analyzed after each week (week 1-week 5) by one-way ANOVA, at each time point the HVR RUN group ran longer than the WT RUN group, which was followed by the LVR RUN group (HVR RUN>WT RUN>LVRRUN) (**Fig. 2 b**). The difference between each group on each of the weekly time points was found to be significant.

Body Weight and Muscle Weight Data:

Measurements of body mass were recorded weekly throughout the progression of this experiment. Overall, the observed body weights tended to be highly similar between the groups. The initial measurement of body mass at 28-34 days of age indicated there was

not a statistically significant difference between the HVR, LVR, and WT lineages. The final measurement of body mass was not significant.

Food Intake Data:

Measurements of food intake were taken weekly throughout the progression of this experiment (**Fig. 3**). The initial food intake measurement was taken 1 week into the study and there was not significance found between the groups.

At week 2 there were several significant differences. This study found that the HVR line's food intake was significantly greater than both the WT line ($P = <0.001$) and the LVR line ($P = <0.001$). Additionally, the HVR RUN ($P = 0.001$) and the WT RUN ($P = <0.001$) groups had a significantly higher food intake than their group's SED counterparts. In the LVR line, the LVR SED had a significantly greater food intake than the LVR RUN ($P = 0.002$). Amongst the SED group, the HVR SED ($P = 0.014$) and the LVR SED ($P = 0.003$) had a significantly higher food intake than the WT SED. Amongst the RUN group, the HVR RUN had a significantly greater food intake than the WT RUN ($P = 0.015$) and the LVR RUN ($P = <0.001$) and the WT RUN had a significantly greater food intake than the LVR RUN ($P = 0.002$).

At week 3 several significant differences were observed. The HVR line was found to have a significantly higher food intake than the LVR line ($P = 0.047$). Further, the HVR RUN had a significantly higher food intake than the HVR SED ($P = <0.001$) and the WT RUN had a significantly higher food intake than the WT SED ($P = <0.001$). At week 3, the LVR SED no longer had a significantly higher food intake than the LVR RUN.

Within the RUN group, both the HVR RUN ($P = <0.001$) and the WT RUN ($P = <0.001$) had a significantly higher food intake than the LVR RUN group.

At week 4, The HVR line ($P = <0.001$) and the WT line ($P = 0.018$) had a significantly higher food intake than the LVR line. Within their respective lines, the HVR RUN had a significantly higher food intake than the HVR SED ($P = <0.001$) and the WT RUN had a significantly higher food intake than the WT SED ($P = <0.001$). Amongst the RUN group, both the HVR RUN ($P = <0.001$) and the WT RUN ($P = <0.001$) had a greater food intake than the LVR RUN.

Finally, at week 5, there were several significant differences. However, due to missing data, the HVR RUN and WT RUN groups were not included. Despite this limitation, there was still significant differences amongst the SED group. Amongst the SED group, the LVR SED had a significantly greater food intake than both the HVR SED ($P = 0.33$) and the WT SED ($P = 0.046$).

PGC-1 α

PGC-1 α has been well established as the master regulator of mitochondrial biogenesis. Exercise is an important non-pharmacological strategy for increasing mitochondrial biogenesis and, thereby, preventing mitochondrial dysfunction and its associated diseases. For this reason we first analyzed the density of PGC-1 α protein with Western analysis in the NAc, LH, and HC. Due to differences in the appearances of the PGC-1 α protein bands between the two separate antibodies, it was determined that the two separate antibodies used could not be quantified together in a manner that meets the standards of this lab because the SantaCruz PGC-1 α antibody gave two thin bands while

the Millipore PGC-1 α antibody gave one thicker band. For this reason, the initial LVR and HVR groups that used the Millipore PGC-1 α and the WT and LVR group using the SantaCruz PGC-1 α were quantified independently of each other.

Analysis of PGC-1 α Protein HVR vs. LVR

Using a two-way ANOVA analysis it was established that protein levels of PGC-1 α between the LVR sedentary and HVR sedentary lines were not significantly different in the NAc

(**Fig. 5**). In the HC, PGC-1 α protein in the LVR line was significantly higher (1.27-fold greater) than the HVR line (P= 0.047) (**Fig. 6**). Further, in the LH, the SED group was significantly higher (1.29-fold greater) in PGC-1 α protein content than the RUN groups (**Fig. 7**), which was atypical of accepted physiological responses to exercise training (P= 0.035).

Analysis of PGC-1 α Protein LVR vs. WT

Protein analysis of Western blots from the LVR and WT portion (which differs from comparisons of LVR to HVR in fig. 3) of the experiment were carried out utilizing two-way ANOVA analysis. In the NAc, no significant differences between the LVR and WT were found in PGC-1 α protein levels (**Fig. 8**). Similarly, there were no significant differences in PGC-1 α protein between the WT and LVR rats in the HC or the LH tissues (**Fig. 9-10**).

Analysis of PGC-1 α HVR vs. WT

Discrepancies between the Millipore PGC-1 α antibody, used in the HVR vs. LVR experiment, and the SantaCruz antibody, used in the LVR vs. WT experiment, prevented comparison of HVR to WT rats.

Analysis of OXPHOS Proteins

Levels of proteins in the mitochondrial oxidative phosphorylation (OXPHOS) system were determined in the NAc, LH, and HC through Western blot analysis for all five complexes in OXPHOS, which allows for a thorough analysis of the effects of genes and environmental interactions (e.i., inherent genes producing voluntary running distance and responsiveness of other inherent genes for mitochondrial protein level). Although two, separate experiments were performed, the data obtained from the Western blotting analysis was able to be integrated together due to the use of the same OXPHOS antibody and because both experiments utilized the same procedures.

Analysis of OXPHOS Protein in the NAc

In the NAc, several significant changes in OXPHOS proteins were observed. Complex I: Complex I demonstrated a statistically significant interaction between line and treatment (P= 0.049) (**Fig. 11**). Unfortunately the main effects could not properly be interpreted with the Holm-Sidak method of multiple comparison because the statistical analysis software did not recommend further interpretation with this multiple comparison procedure because of unequal distribution, as was used in the rest of the analysis. It should be noted that both tests indicated that there were significant differences, but the Holm-Sidak was unable to properly determine the interactions and the SigmaPlot software advised me to use a different method. Using the Student-Newman-Keuls

method of multiple comparison, it was determined that the fold change of complex I proteins was significantly greater in LVR SEDs than in HVR SEDs (LVR SED were 1.39-fold greater than HVR SED). *Complex II*: In complex II (**Fig. 12**), there were several significant differences observed among the three lines in the NAc. The WT line had significantly greater fold change that was 1.35-fold greater than HVR and 1.24-fold greater than LVR) in complex II proteins [(P= 0.002) and (P= 0.023) respectively.] However, values were not significant between the LVR and HVR lines. Additionally significance was discovered between the treatment groups in complex II. Within the RUN groups, the WT rats demonstrated a significantly greater fold change than HVR rats (WT RUN 1.43-fold greater than HVR RUN) (P= 0.011) and the LVR rats (WT RUN 1.27-fold greater than LVR RUN) (P= 0.044). *Complex III*: There were several significant differences in complex III however due to failures in the Shapiro-Wilk Normality Test and the Brown-Forsythe Equal Variance Test, the data was rendered statistically invalid. Therefore, the data was transformed by applying a logarithm. Transforming data using a logarithm is a widely-accepted statistical procedure used to make highly skewed distributions less skewed, which enables it to stabilize variance and creates a normal distribution while preserving the location of statistics such as the median (Kreutz et al., 2007). After this transformation both normality and equal variance were passed. Nonetheless, there were no statistically significant differences found in the data obtained from complex III (**Fig. 13**). *Complex IV*: In complex IV (**Fig. 14**), the fold change of OXPHOS protein was significantly greater in the WT lines than HVR lines (WT 1.48-fold greater than HVR) (P= 0.006). Further, the fold change of protein was significantly greater in the WT lines than HVR lines within the SED group was observed

(WT SED 1.77-fold greater than HVR SED) (P= 0.003). Complex V: There were no statistically significant differences found in the data obtained from complex V (**Fig. 15**).

Analysis of OXPHOS Protein in the HC

Complex I: In the HC, there were significant differences among the lines as a result of treatment in complex I (P= 0.041) (**Fig. 16**). This led to testing for group differences among lines. However, the multiple comparison procedures used were not able to isolate the interactions between the groups, and as a result the significance was not able to be properly determined. Complex II: In complex II the SED treatment groups had significantly larger fold changes in protein than the RUN groups (SED 1.24-fold greater than RUN) (P= 0.018) (**Fig. 17**). The WT SED group were observed to have a significantly larger fold change of complex II proteins than the WT RUN group (WT SED 1.62-fold greater than WT RUN) (P= 0.002). Also, within the SED groups, the WT rats had higher fold changes in complex II proteins than the HVR rats (WT SED 1.46-fold greater than HVR SED) (P= 0.027) and the LVR (WT SED 1.52-fold greater than LVR SED) (P= 0.028). Complex III: In complex III (**Fig. 18**), the LVR line was observed to have a significantly larger fold change in protein than the HVR line (LVR 1.69-fold greater than HVR) (P= 0.012). Also, within the LVR group the SED animals had a greater change than the RUN (LVR SED 1.93-fold greater than LVR RUN) (P= 0.002). This trend continued as the LVR SED also possessed a significantly greater fold change of complex III proteins than both the HVR SED (LVR SED 2.58-fold greater than HVR SED) (P= <0.001) and the WT SED (LVR SED 1.57-fold greater than WT SED) (P= 0.040). Complex IV: No significant changes were found in complex IV proteins potentially due to large variations in WT SED and WT RUN (**Fig. 19**). Complex V: In

complex V (**Fig. 20**), both the LVR (LVR 1.86-fold greater than HVR) ($P= 0.001$) and the WT (WT 1.81-fold greater than HVR) ($P= 0.002$) were significantly greater than the HVR lines. This same effect occurred within the SED group as both the WT (WT SED 2.55-fold greater than HVR SED) ($P= <0.001$) and the LVR (LVR SED 2.45-fold greater than HVR SED) ($P= 0.001$) were observed to have greater fold change in complex V proteins than the HVR rats.

Analysis of OXPHOS Proteins in the LH

Analysis of OXPHOS proteins in the LH, a region of the brain responsible for energy regulation and integration/modulation of cardiorespiratory responses during exercise revealed several significant changes in OXPHOS proteins. Complex I: In complex I (**Fig. 21**), the LVR line presented a higher level of protein than the WT line (LVR 1.51-fold greater than WT) ($P= 0.011$). Complex II: No significant differences were observed in complex II (**Fig. 22**). Complex III: In complex III (**Fig. 23**), it was observed that both the LVR line (LVR 6.79-fold greater than WT) ($P= <0.001$) and the HVR line (HVR 4.43-fold greater than WT) ($P= <0.001$) had a higher fold change than the WT. Further, within the SED groups, both the LVR (LVR SED 10.7-fold greater than WT SED) ($P= <0.001$) and the HVR (HVR SED 6.33-fold greater than WT SED) ($P= <0.001$) had a higher fold change than the WT SED group. Complex IV: There were no significant changes observed in complex IV proteins (**Fig. 24**). Complex V: In complex V (**Fig. 25**), there was a significantly greater fold change of protein amongst SED animals when compared to those in the RUN group (SED 1.26-fold greater than RUN) ($P= 0.011$). This trend was paralleled amongst the LVRs (LVR SED 1.34-fold greater than LVR RUN) ($P= 0.032$).

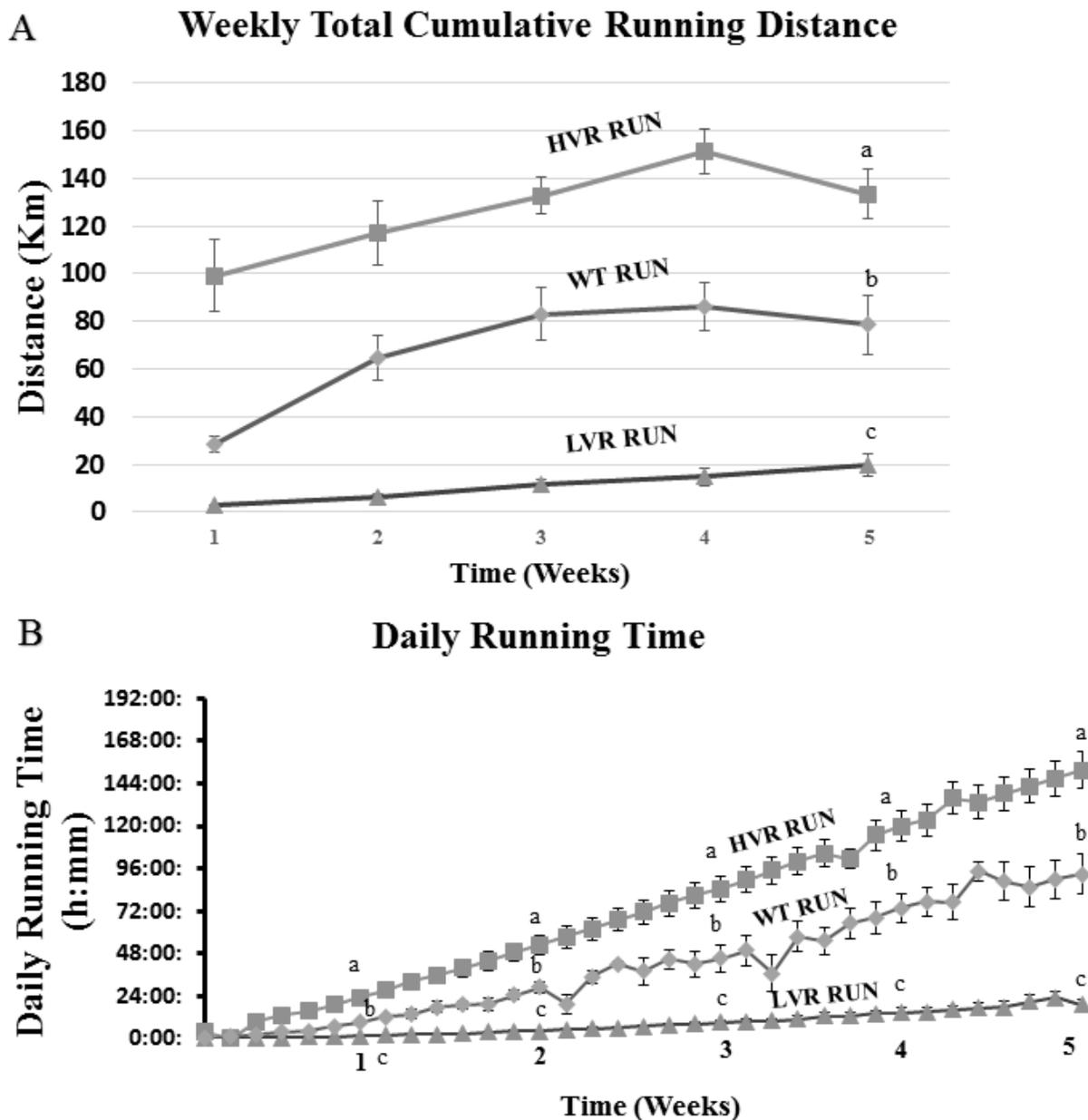


Fig. 2. Differences in Running.

The square indicators represent the HVR RUN group, the diamond indicators represent the WT RUN group, and the triangle indicators represent the LVR RUN group. (A) HVR RUN rats ran significantly farther distances the WT RUN, which also ran significantly farther distances than the LVR RUN rats. This is indicated by the letters a, b, and c. (B) Note a linear trend as each group slowly increases the duration of their daily running. At each time point (week 1- week 5) the letters a, b, and c indicate significantly different

durations of running between the 3 groups.

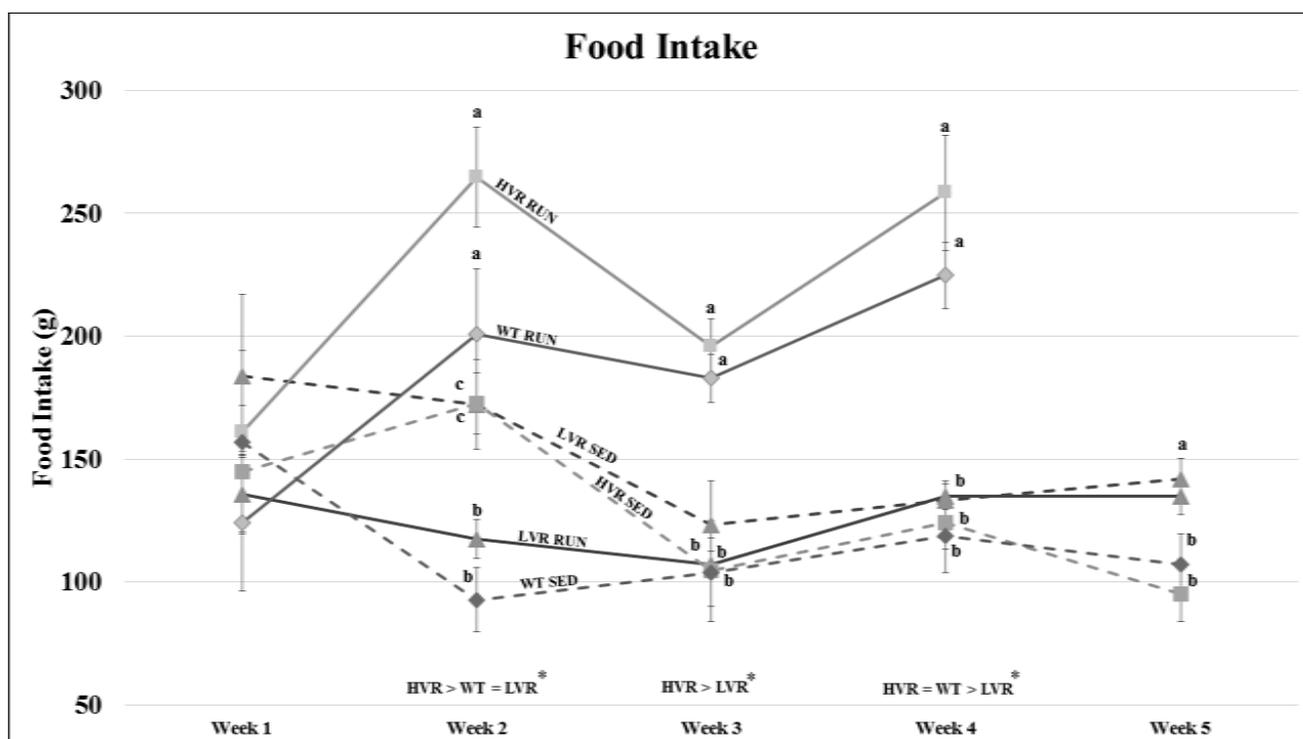


Fig. 3. Food Intake.

The solid lines represent the RUN group and the dashed lines represent the SED group. The square indicator represents the HVR group, the diamond indicator represents the WT group, and the triangle indicator represents the LVR group. For instance the square indicator on the solid line represents the HVR RUN group, whereas the square indicator on the dashed line represents the HVR SED group. Differences in assigned letters (a-c) represent significant differences. At the bottom of the chart above the x-axis the line significance is given. At week 5, due to missing data the HVR RUN and WT RUN were excluded. *Definitions: HVR includes HVR RUN and HVR SED, WT includes WT RUN and WT SED, and LVR includes LVR RUN and LVR SED.

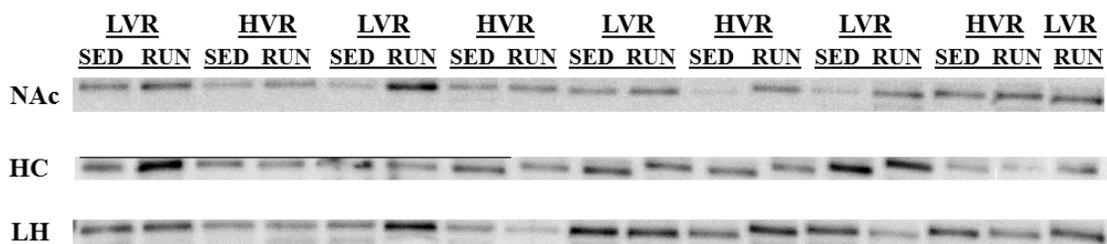


Fig. 4. Representative Blot for PGC-1 α (LVR vs. HVR)

This is a representative blot of the bands that were analyzed at approximately 113 Kda as PGC-1 α . Region of the brain is indicated on the left and above the bands is indicated their respective lineage and grouping.

PGC-1α Protein Summary		
	LVR > HVR	LVR = WT
Nucleus Accumbens	N.S.	N.S.
Ventral Hippocampus	1.27-fold (P=0.047)	N.S.
Lateral Hypothalamus	SED > RUN 1.29-fold (P= 0.035)	N.S.

N.S. = Not Significant

Table 1. Summary of PGC-1 α Protein

This table gives a summary of the responses of the three different rat-lines in regards to PGC-1 α protein expression in the NAc, HC, and LH. The abbreviation N.S. was used for not significant.

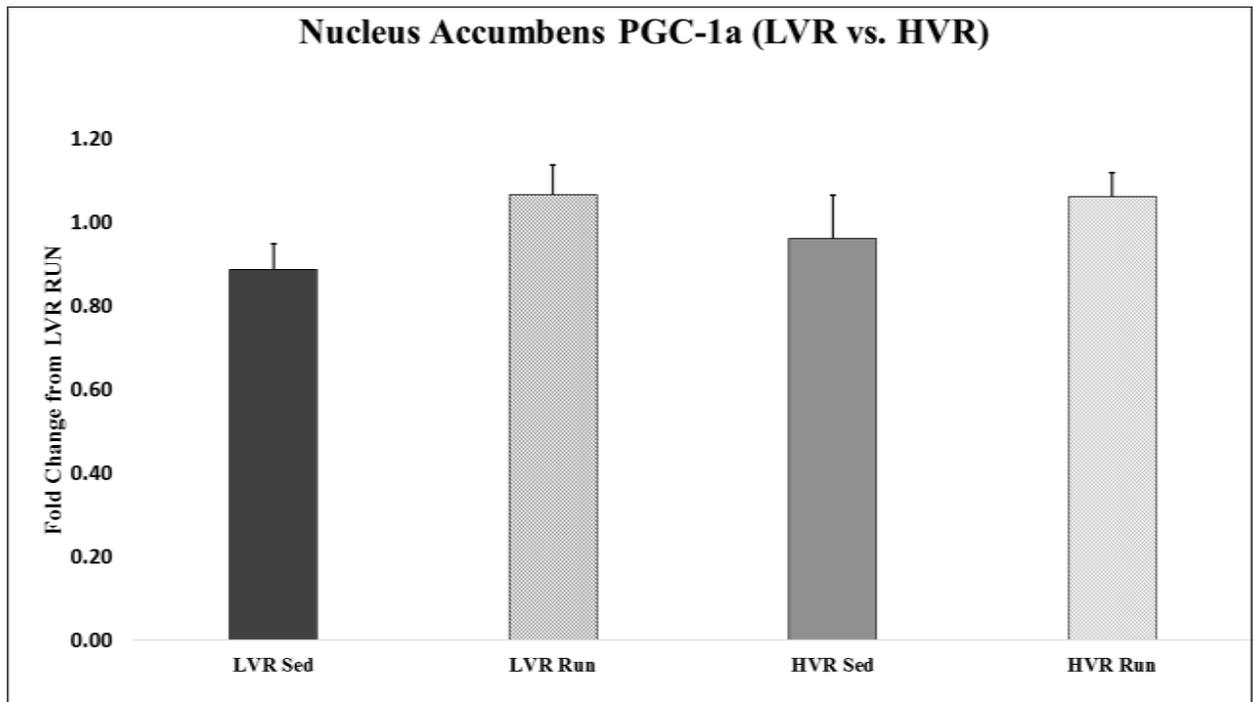


Fig. 5. PGC-1 α Protein Quantification in the NAc (LVR vs. HVR).

Bars represent the concentration of PGC-1 α protein as a fold change, normalized by LVR RUN protein values. In the NAc, there were no significant differences observed.

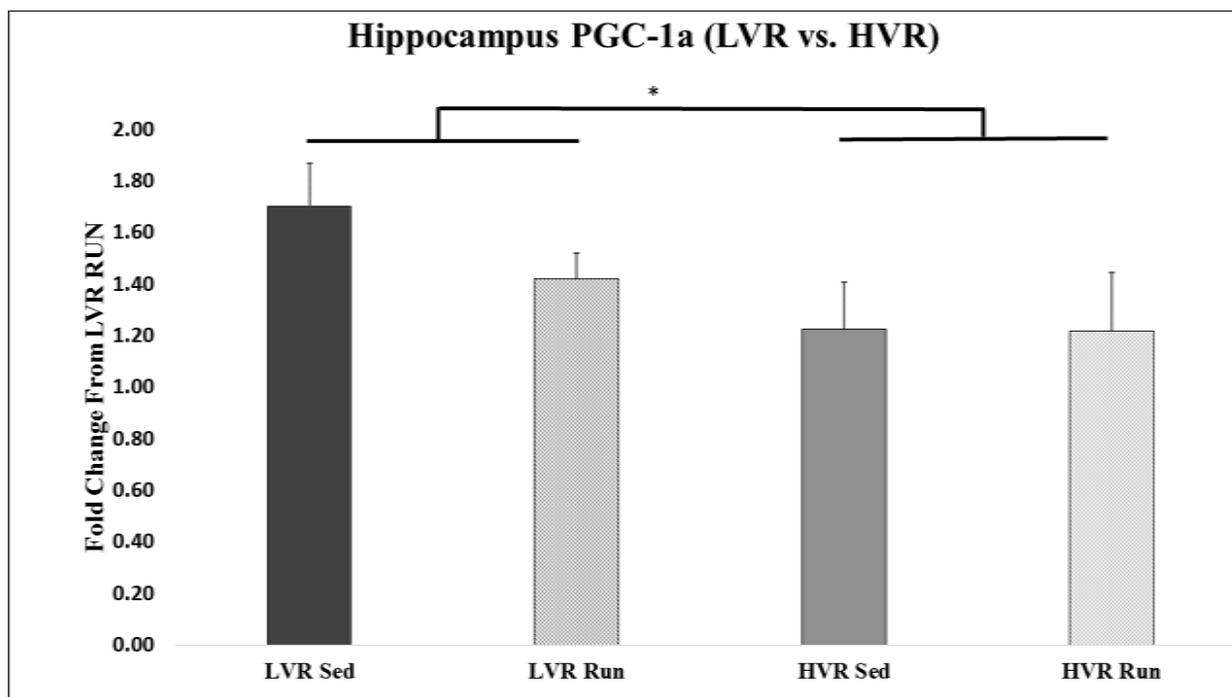


Fig. 6. PGC-1 α Protein Quantification in the HC (LVR vs. HVR).

Bars represent the concentration of PGC-1 α protein as a fold change, normalized by LVR RUN protein values. The asterisk represents significant differences in the fold change of the protein values. In the ventral hippocampus, the LVR line was observed to be 1.27-fold greater than the HVR line ($P=0.047$).

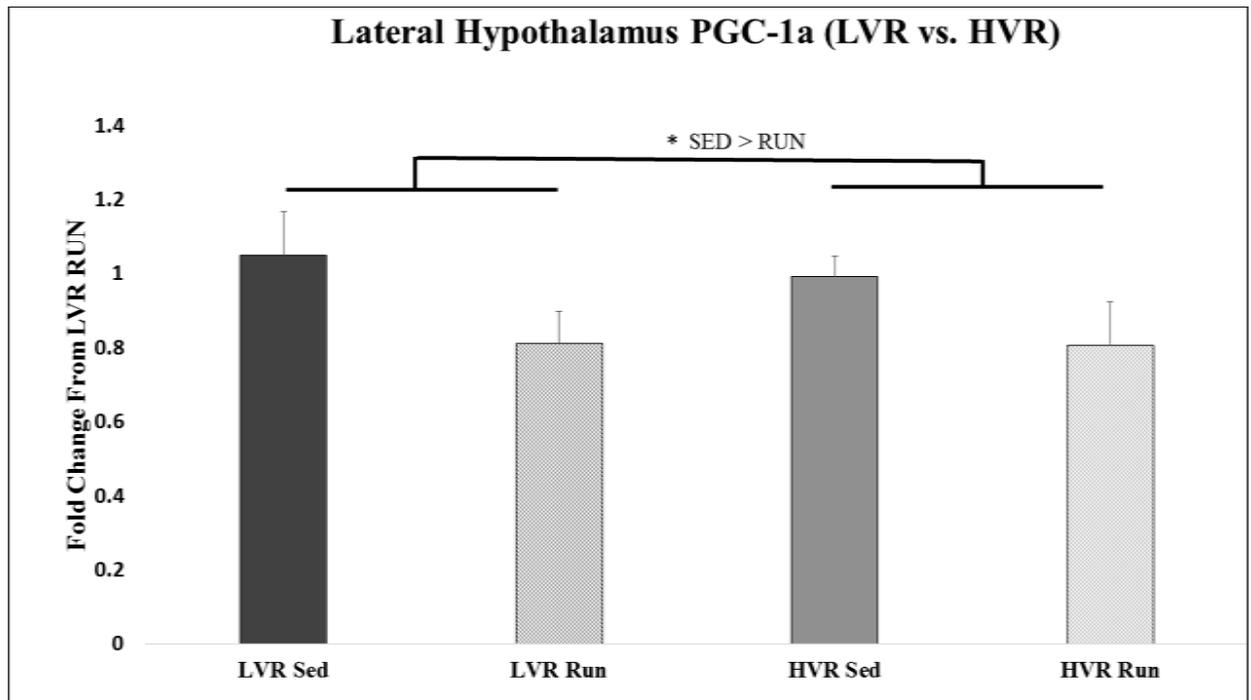


Fig. 7. PGC-1 α Protein Quantification in the LH (LVR vs. HVR).

Bars represent the concentration of PGC-1 α protein as a fold change, normalized by LVR RUN protein values. The asterisk represents significant differences in the fold change of the protein values. In the LH, the SED group demonstrated a 1.29-fold larger protein value than the RUN group ($P=0.035$).

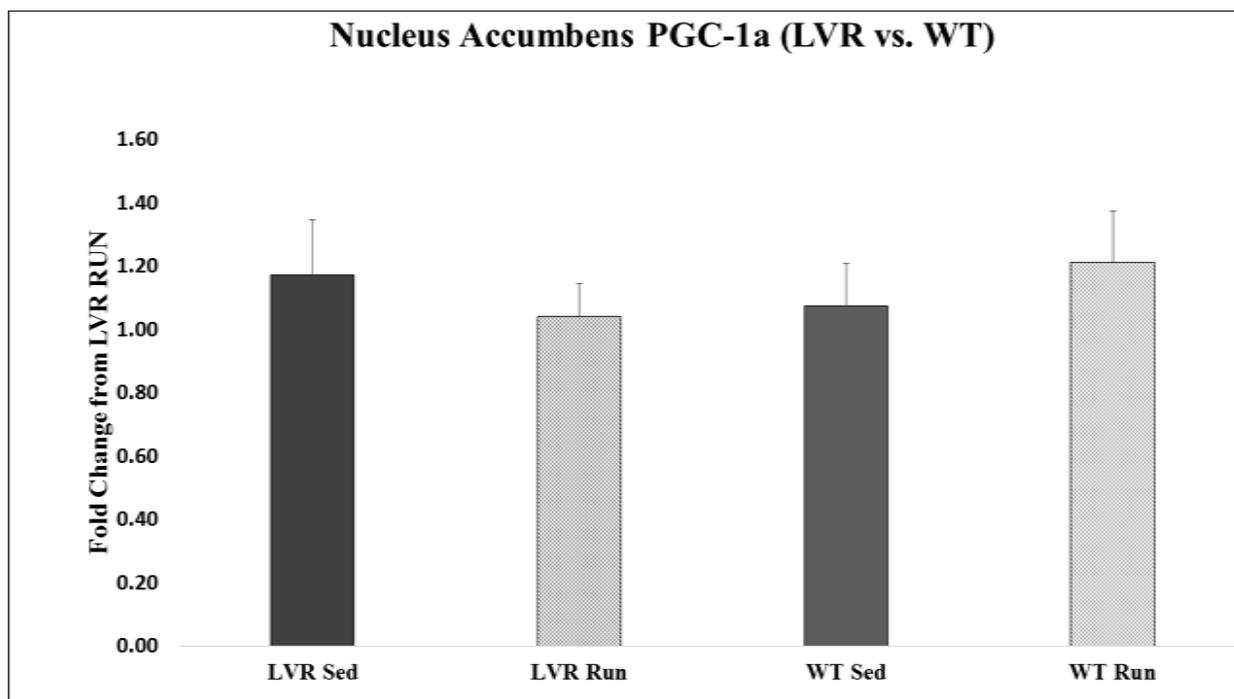


Fig. 8. PGC-1 α Protein Quantification in the NAc (LVR vs. WT).

Bars represent the concentration of PGC-1 α protein as a fold change, normalized by LVR RUN protein values. In the NAc, there were no significant differences observed.

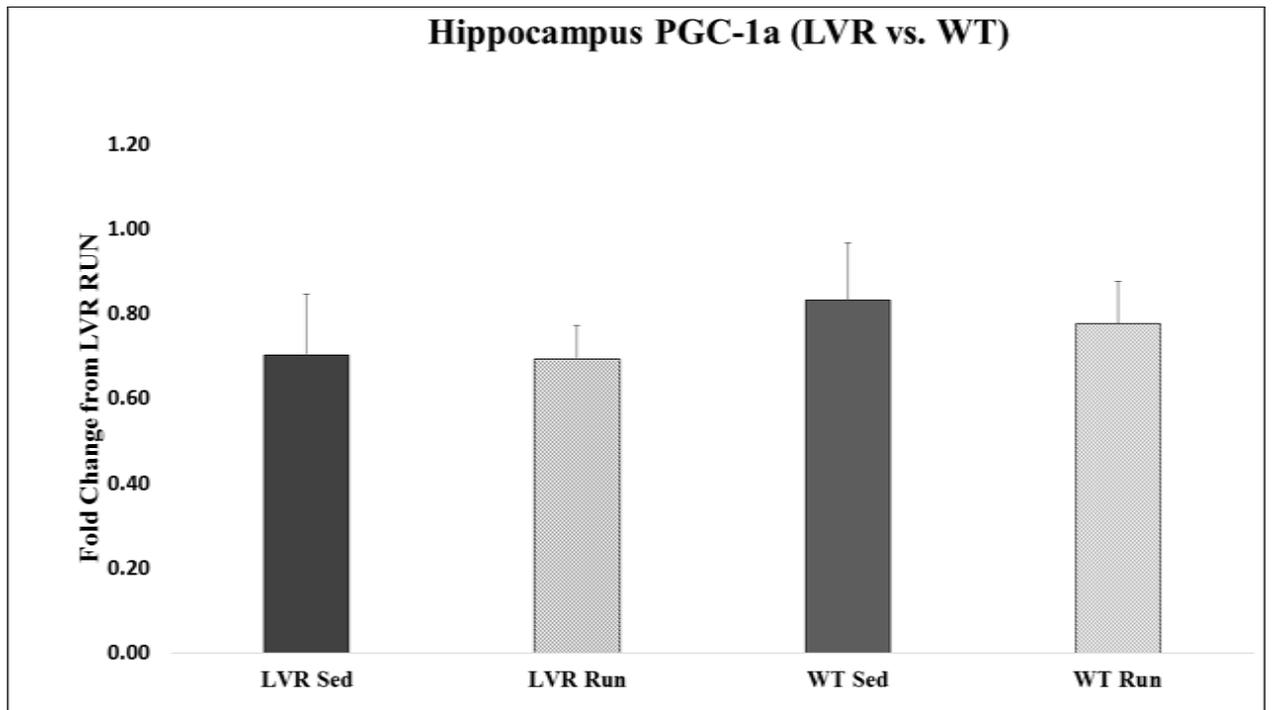


Fig. 9. PGC-1 α Protein Quantification in the HC (LVR vs. WT).

Bars represent the concentration of PGC-1 α protein as a fold change, normalized by LVR RUN protein values. In the HC, there were no significant differences observed.

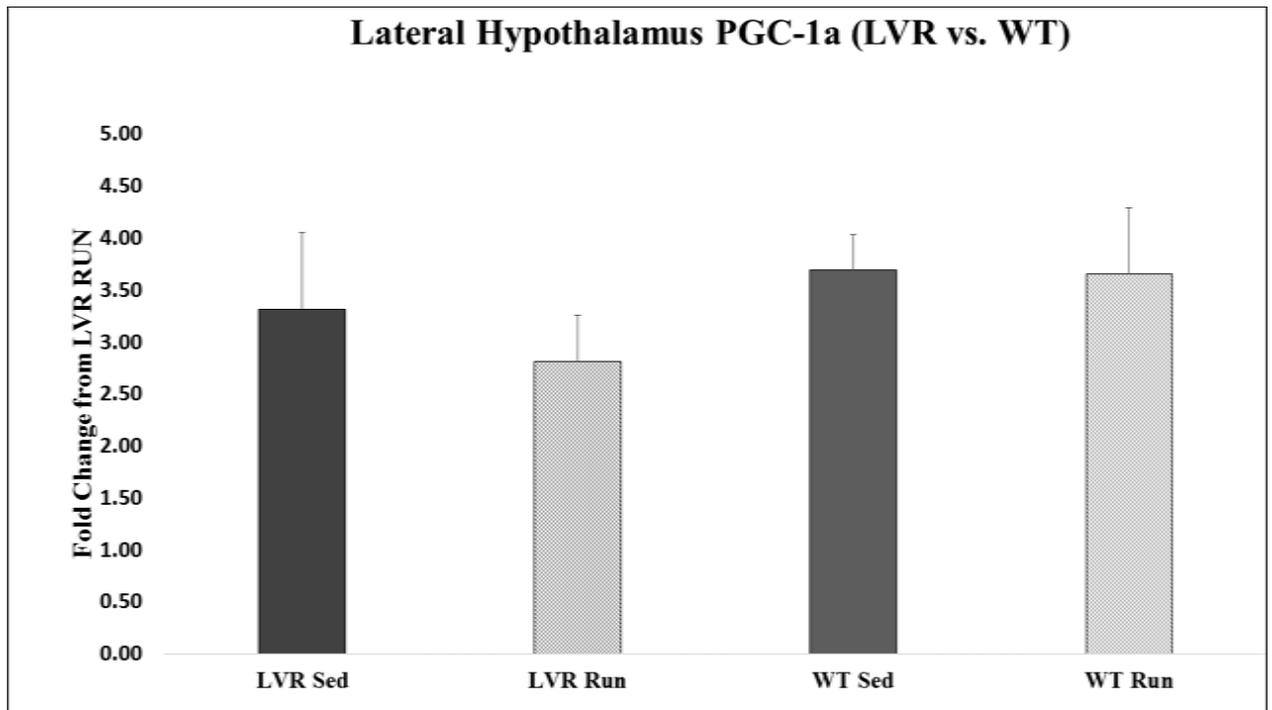


Fig. 10. PGC-1 α Protein Quantification in the LH (LVR vs. WT).

Bars represent the concentration of PGC-1 α protein as a fold change, normalized by LVR RUN protein values. In the LH, there were no significant differences observed.

Electron Transport Chain Protein Complexes					
	I	II	III	IV	V
Nucleus Accumbens	LVR SED > HVR SED (1.39-Fold)	WT > HVR = LVR WT > HVR (1.35-Fold) WT > LVR (1.24-Fold)	N.S.	WT > HVR (1.48 Fold)	N.S.
Ventral Hippocampus	N.S.	WT SED > LVR SED = LVR RUN = HVR SED = HVR RUN = WT RUN	LVR SED > LVR RUN = HVR RUN = WT SED = WT RUN > HVR SED	N.S.	LVR SED = WT SED > LVR RUN = HVR RUN = WT RUN > HVR SED
Lateral Hypothalamus	LVR > WT	N.S.	LVR > WT (6.79-Fold) HVR > WT (4.43-Fold) LVR SED > WT SED (10.7-Fold) HVR SED > WT SED (6.33-Fold)	N.S.	SED > RUN (1.26-Fold)

Table 2. Summary of Electron Transport Chain Protein Complexes

This table gives a summary of the responses of the three different rat-lines in regards to OXPHOS complex proteins in the NAc, HC, and LH. The abbreviation N.S. was used for not significant.

		Complex I	Complex II	Complex III	Complex IV	Complex V
Nac	HVR	↓	—	—	↓	—
	WT	—	↑	—	↑	—
	LVR	↑	—	—	—	—
HC	HVR	—	—	↓	—	↓
	WT	—	↑	—	—	↑
	LVR	—	—	↑	—	↑
LH	HVR	—	—	↑	—	—
	WT	↓	—	—	—	—
	LVR	↑	—	↑	—	↑

Table 3. Generalized Summary of Trends in the Electron Transport Chain Complex Proteins.

This table gives a generalized summary of trends in the electron transport chain complex proteins. The direction of the up and down arrows is relative to group(s) with the horizontal dash-like line. The up arrow is greater than the group(s) with the line; the down arrow is less than the group(s) with the line for a given complex in a given brain region.

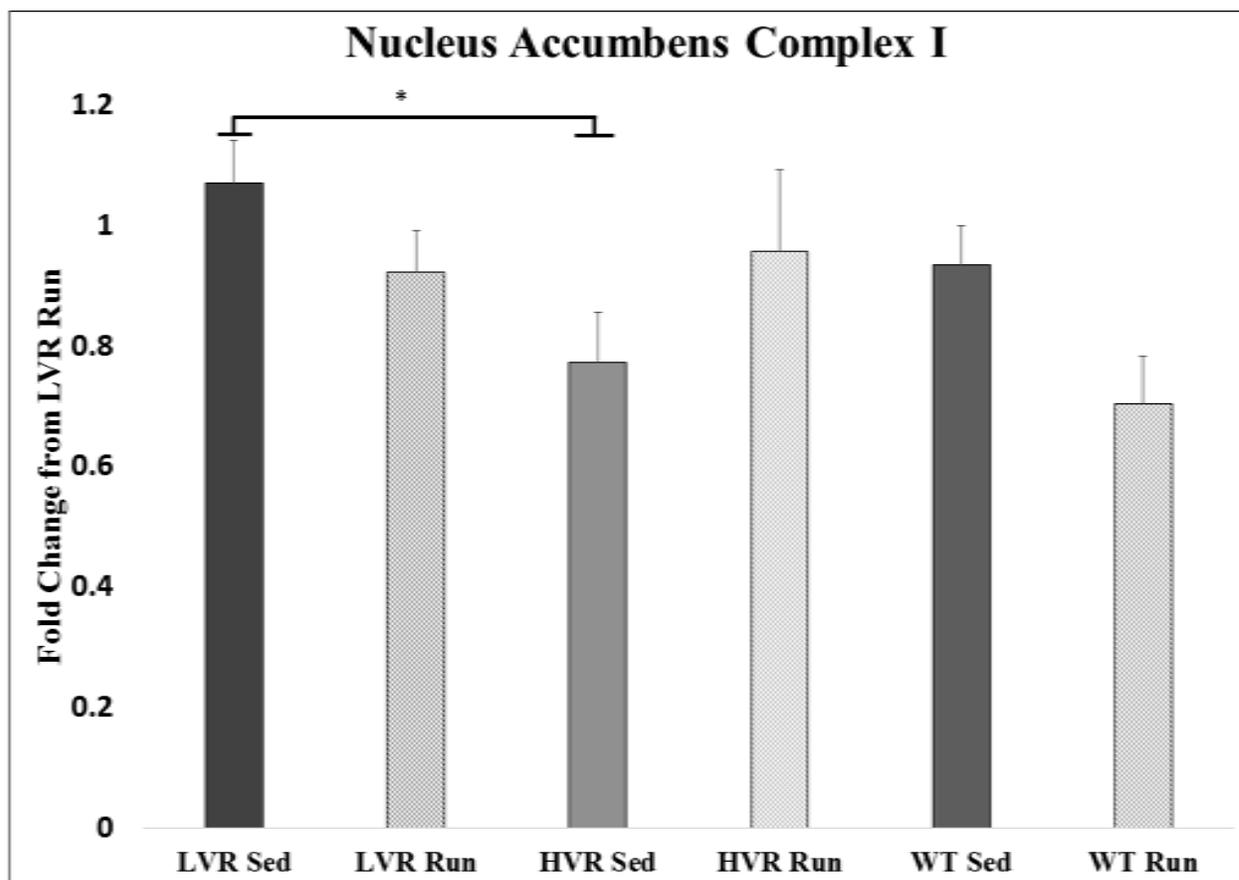


Fig. 11. OXPHOS Complex I Protein Quantification in the NAc.

Bars represent the concentration of OXPHOS complex I protein as a fold change, normalized by LVR RUN protein values. The asterisk represents significant differences in the fold change of the protein values. LVR SED rats demonstrated to be 1.39-fold greater than HVR SED rats ($P=0.049$).

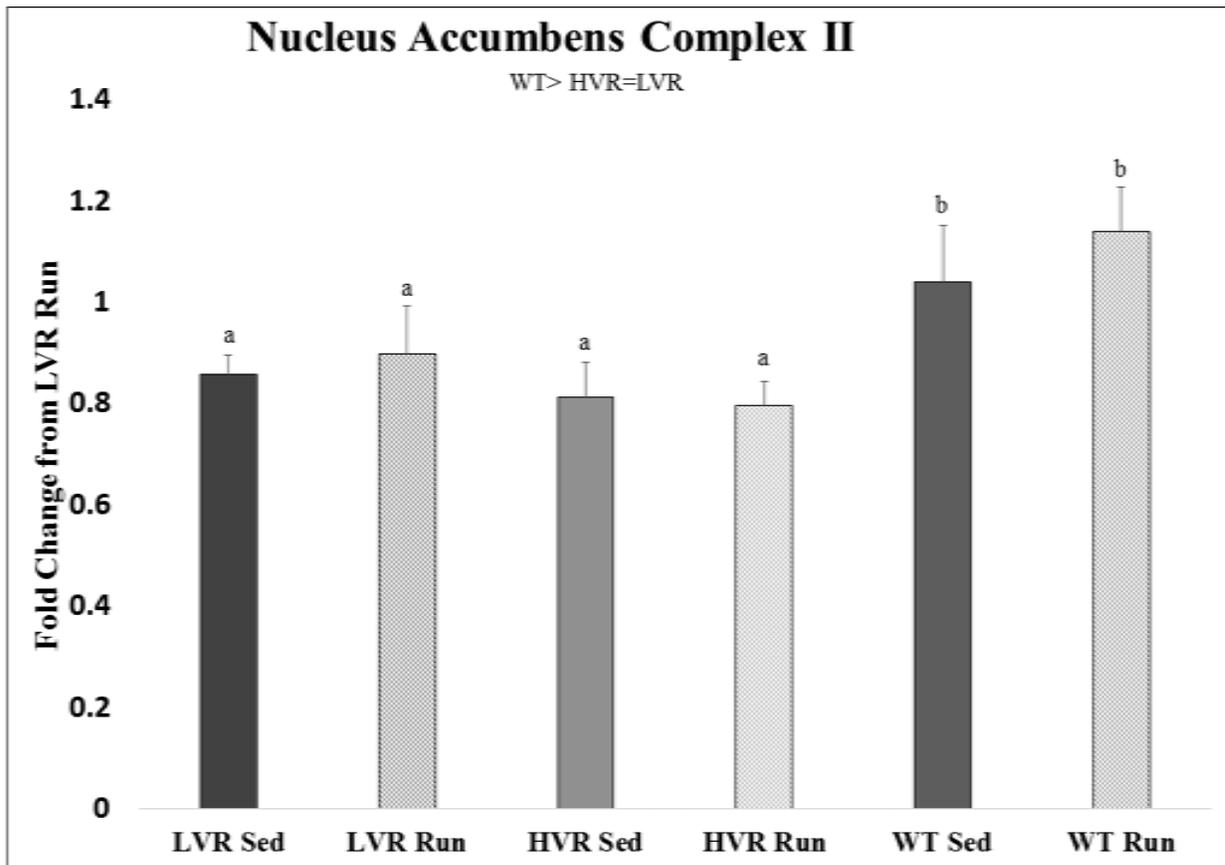


Fig. 12. OXPHOS Complex II Protein Quantification in the NAc.

Bars represent the concentration of OXPHOS complex I protein as a fold change, normalized by LVR RUN protein values. Differences in assigned letters (a-b) also represent significant differences. WT > HVR = LVR. The WT line was 1.35-fold greater than the values of the HVR line and 1.24-fold greater than the LVR line [(P= 0.002) and (P= 0.023) respectively.]

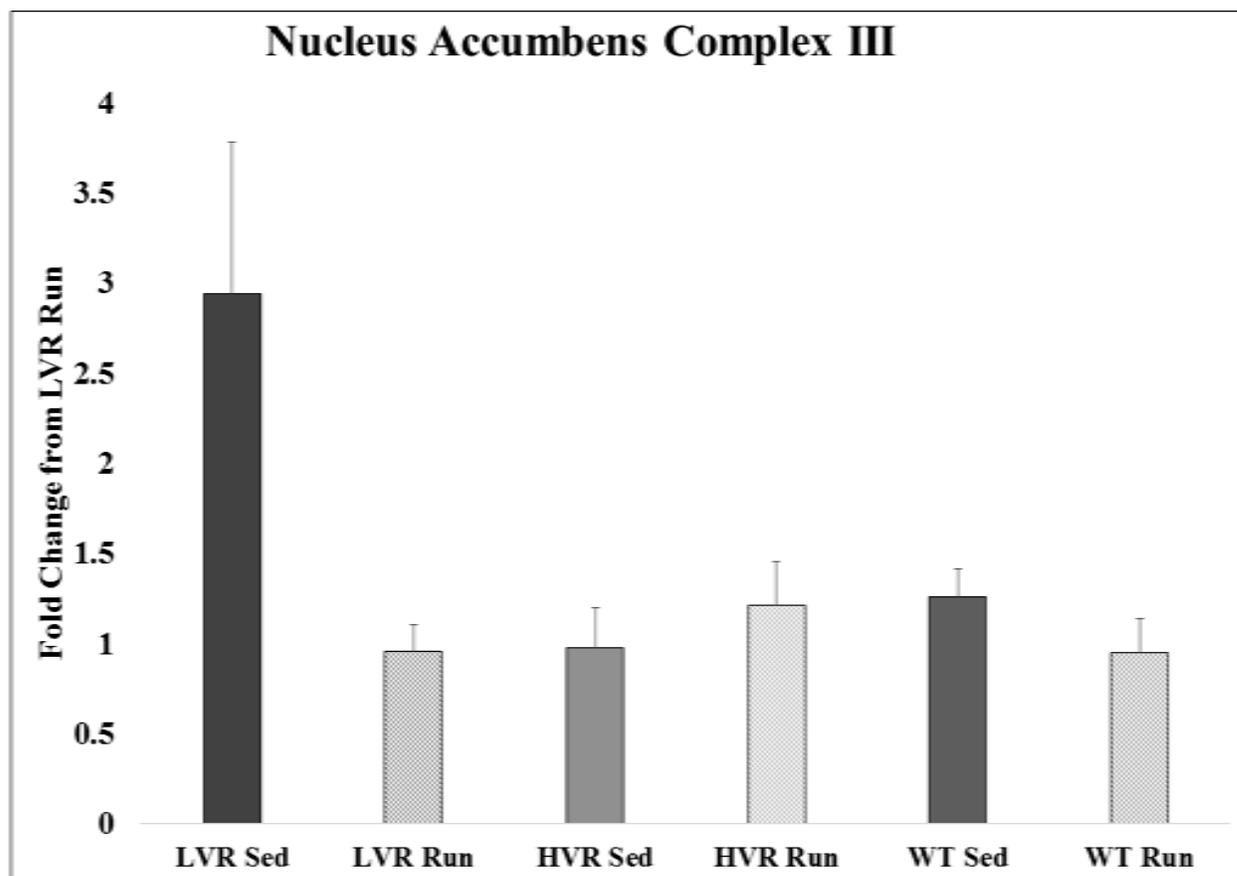


Fig. 13. OXPHOS Complex III Protein Quantification in the NAc.

Bars represent the concentration of OXPHOS complex III protein as a fold change, normalized by LVR RUN protein values. In complex III of the NAc, there were no significant differences observed (see text for explanation).

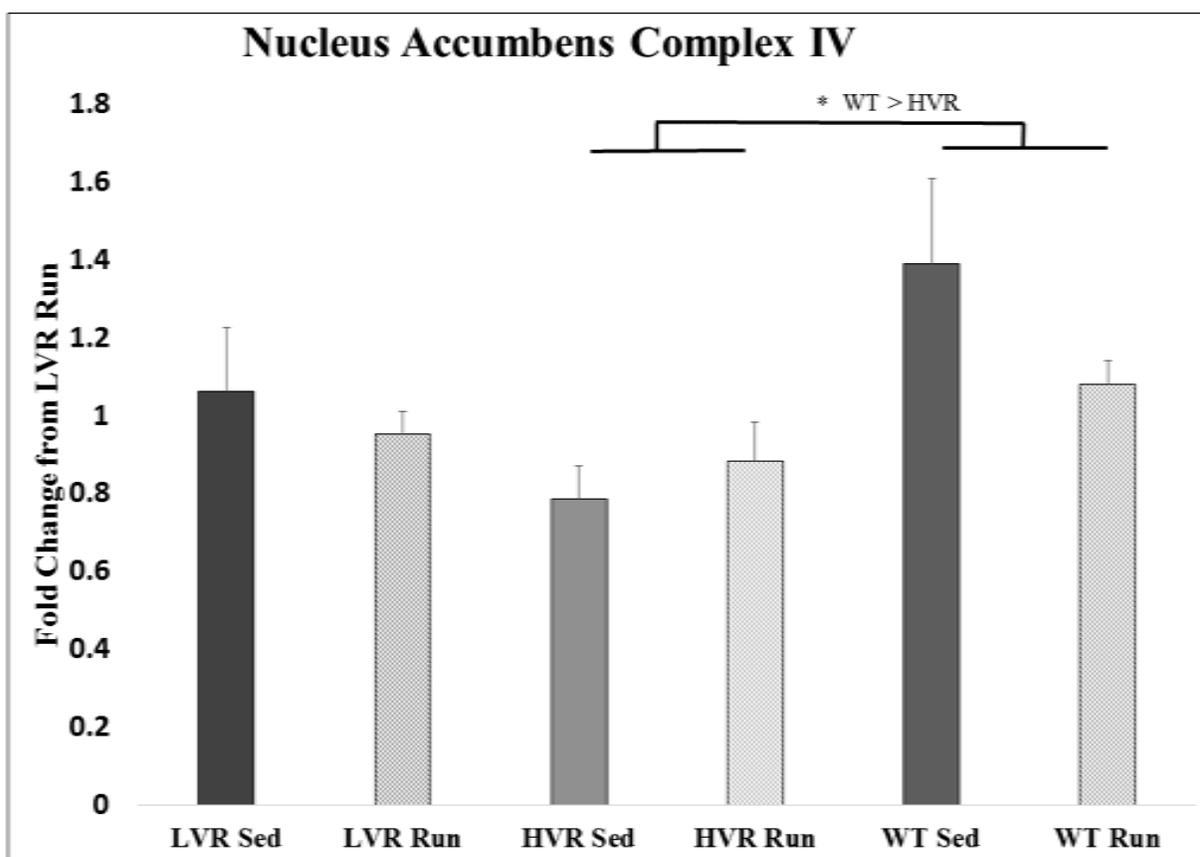


Fig. 14. OXPHOS Complex IV Protein Quantification in the NAc.

Bars represent the concentration of OXPHOS complex I protein as a fold change, normalized by LVR RUN protein values. The asterisk represents significant differences in the fold change of the protein values. The WT line was observed to be 1.48-fold greater than the HVR line (P= 0.006).

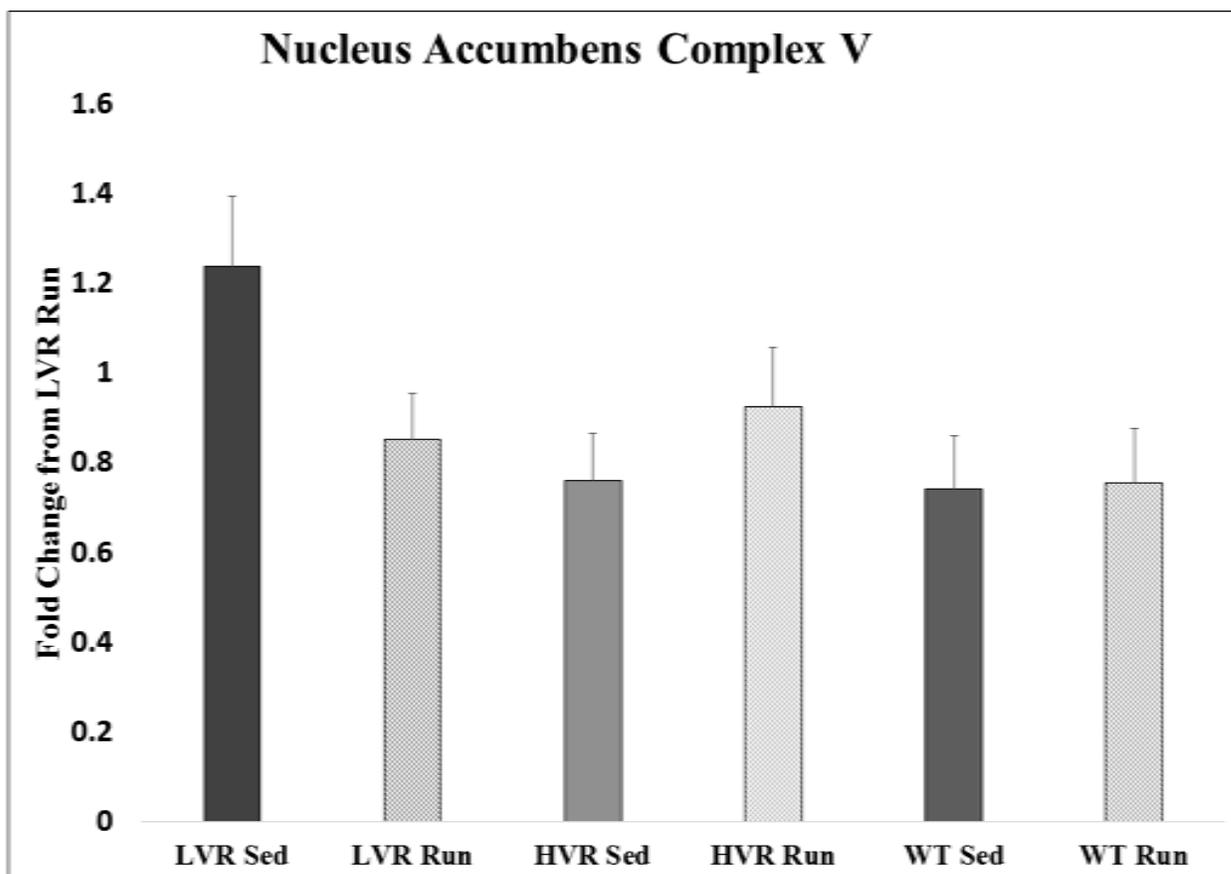


Fig. 15. OXPHOS Complex V Protein Quantification in the NAc.

Bars represent the concentration of OXPHOS complex V protein as a fold change, normalized by LVR RUN protein values. In complex V of the NAc, there were no significant differences observed.

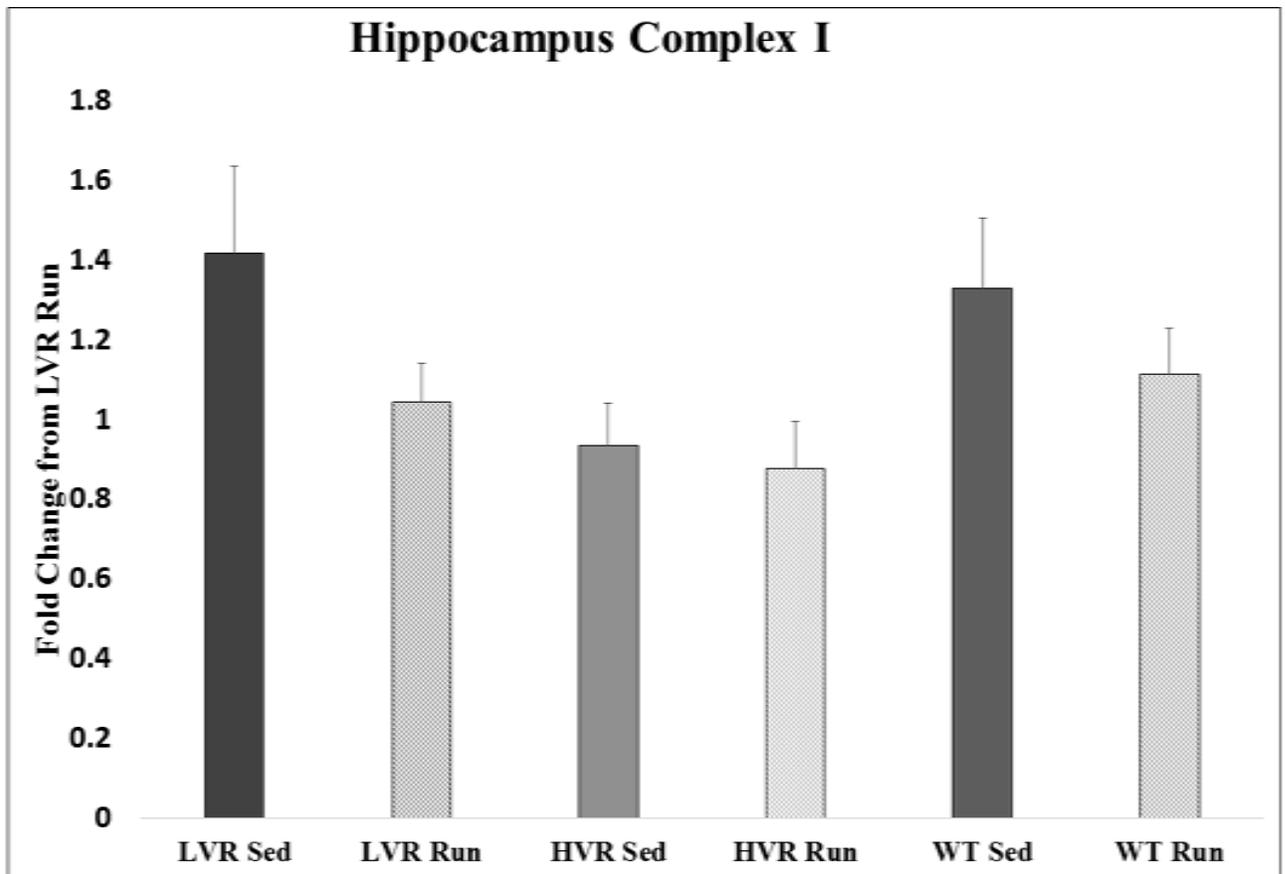


Fig. 16. OXPHOS Complex I Protein Quantification in the HC.

Bars represent the concentration of OXPHOS complex I protein as a fold change, normalized by LVR RUN protein values. In complex I of the HC, there were no significant differences observed.

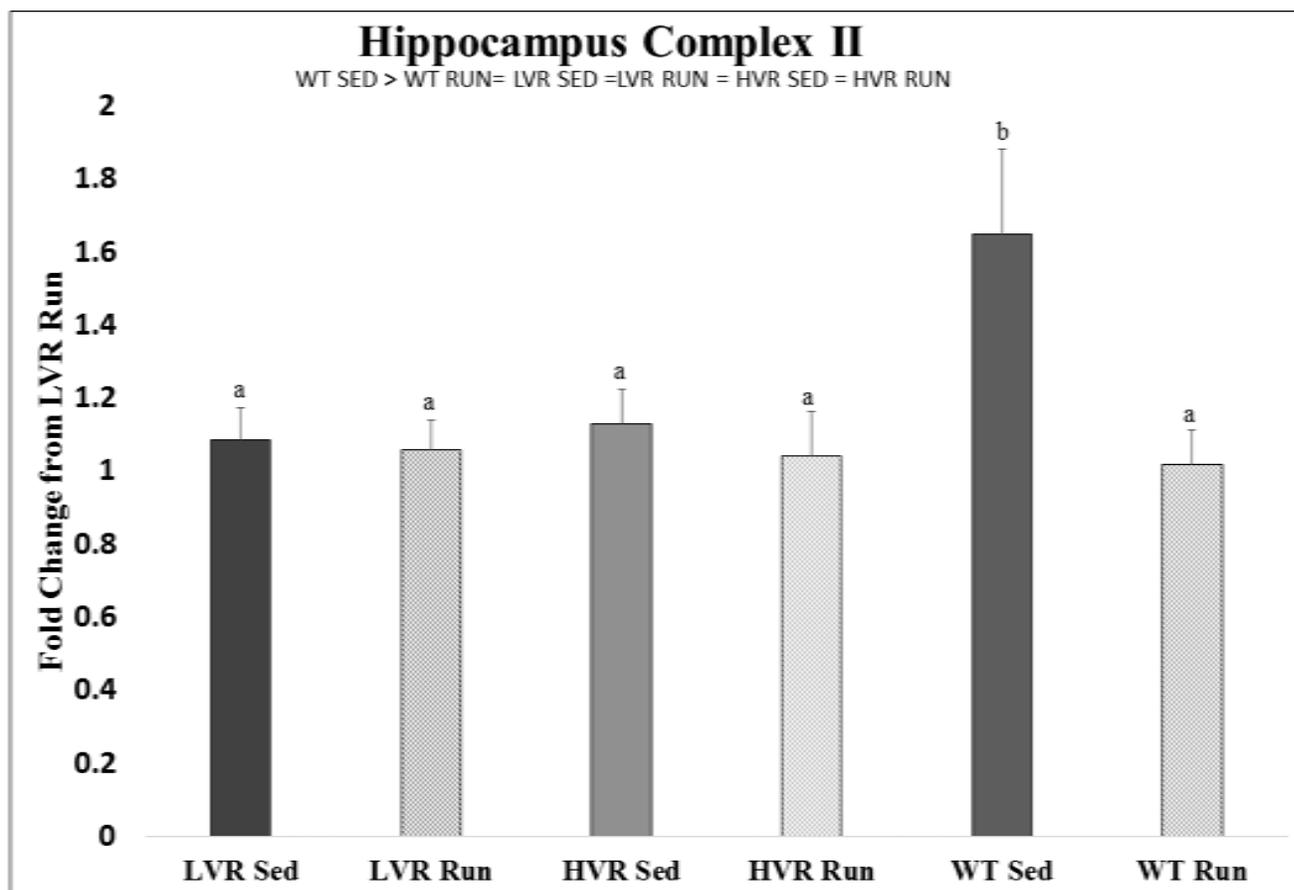


Fig. 17. OXPHOS Complex II Protein Quantification in the HC.

Bars represent the concentration of OXPHOS complex II protein as a fold change, normalized by LVR RUN protein values. Differences in assigned letters (a-b) also represent significant differences. In complex II, SED animals were 1.24-fold greater than RUN ($P= 0.018$). WT SED were 1.62-fold greater than WT RUN, 1.46-fold greater than the HVR SED ($P= 0.027$), and 1.52-fold greater than the LVR SED ($P= 0.028$).

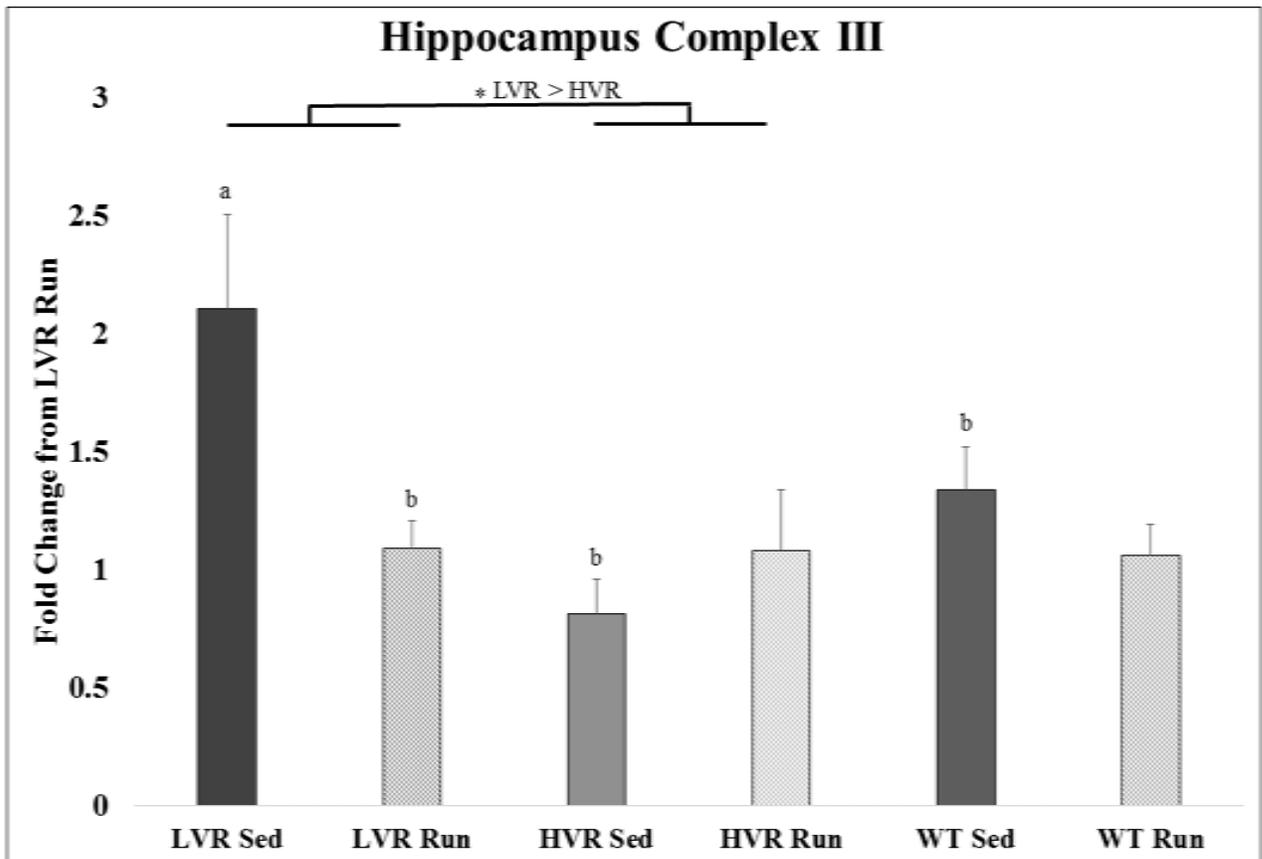


Fig. 18. OXPHOS Complex III Protein Quantification in the HC.

Bars represent the concentration of OXPHOS complex III protein as a fold change, normalized by LVR RUN protein values. The asterisk represents significant differences in the fold change of the protein values. Differences in assigned letters (a-b) also represent significant differences. In complex III, the LVR line had a 1.69-fold greater protein value than the HVR line ($P= 0.012$). The LVR SED group was 1.98-fold greater than the LVR RUN group ($P= 0.002$), 2.58-fold greater than the HVR SED ($P= <0.001$), and 1.57-fold greater than the WT SED ($P= 0.040$), but not greater than HVR RUN or WT RUN.

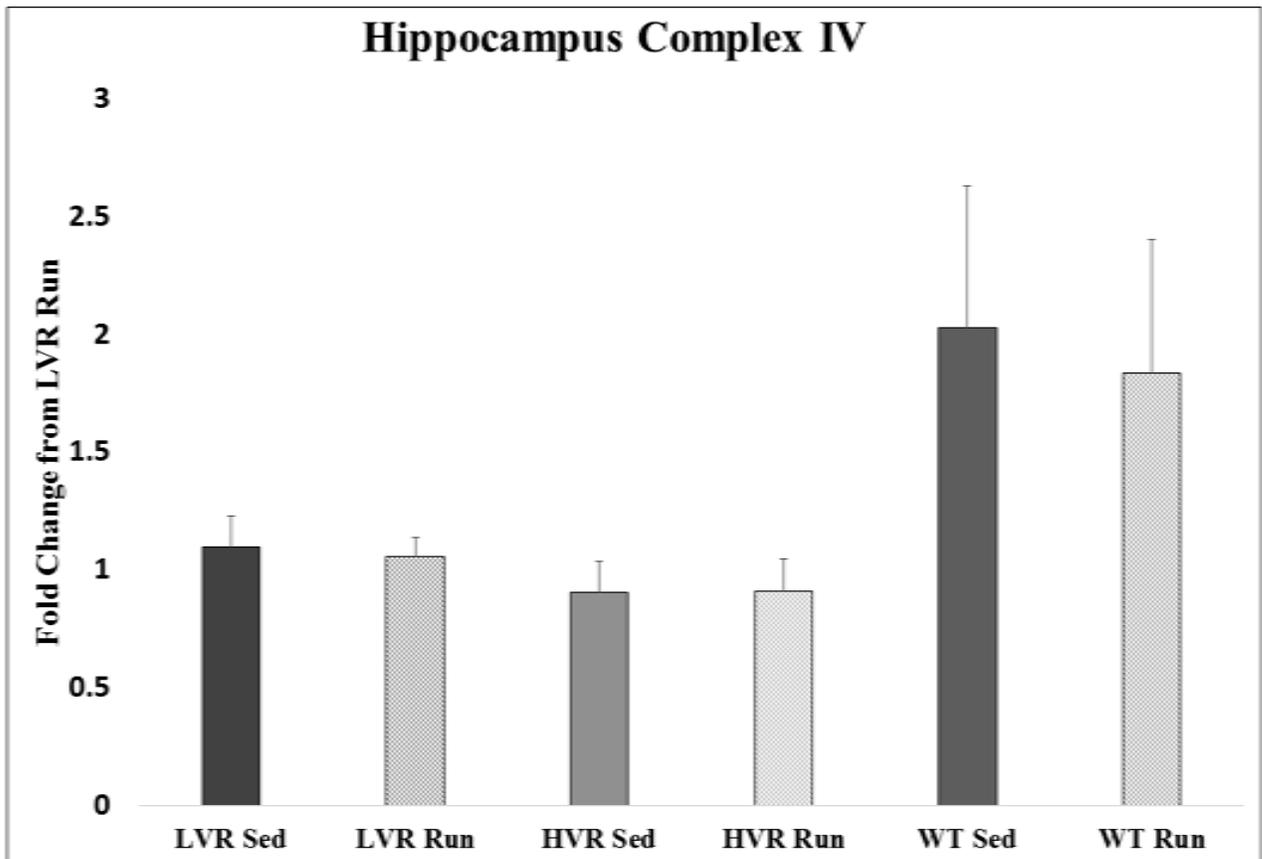


Fig. 19. OXPHOS Complex IV Protein Quantification in the HC.

Bars represent the concentration of OXPHOS complex IV protein as a fold change, normalized by LVR RUN protein values. In complex IV of the HC, there were no significant differences observed, potentially due to large variations in the WT SED and WT RUN groups.

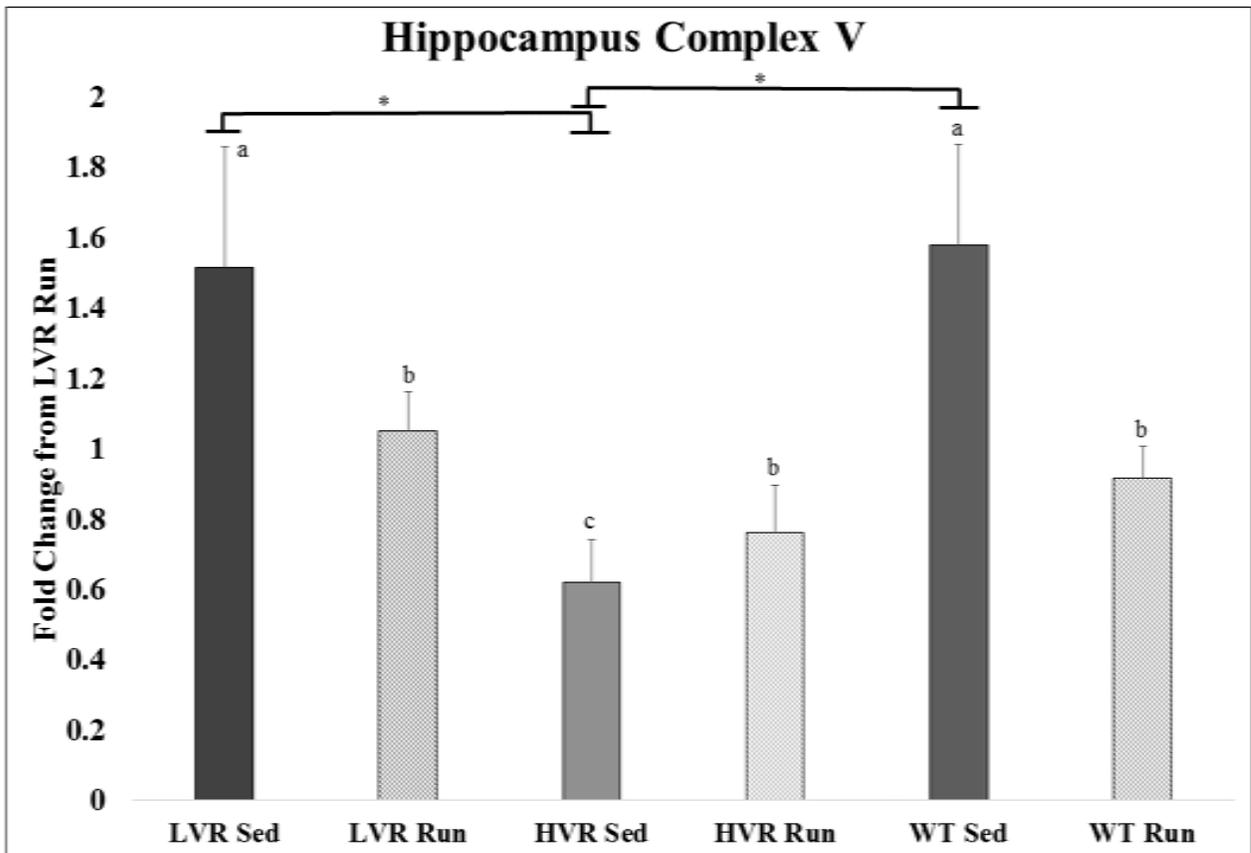


Fig. 20. OXPHOS Complex V Protein Quantification in the HC.

Bars represent the concentration of OXPHOS complex V protein as a fold change, normalized by LVR RUN protein values. The asterisks represent significant differences in the fold change of the protein values. Differences in assigned letters (a-c) also represent significant differences. In complex V, the LVR line was 1.86-fold greater than the HVR line ($P= 0.001$) and the WT line was 1.81-fold greater than the HVR line ($P= 0.002$). The WT SED were 2.55-fold greater than the HVR SED ($P= <0.001$). The LVR SED were 2.45-fold greater than the HVR SED ($P= 0.001$).

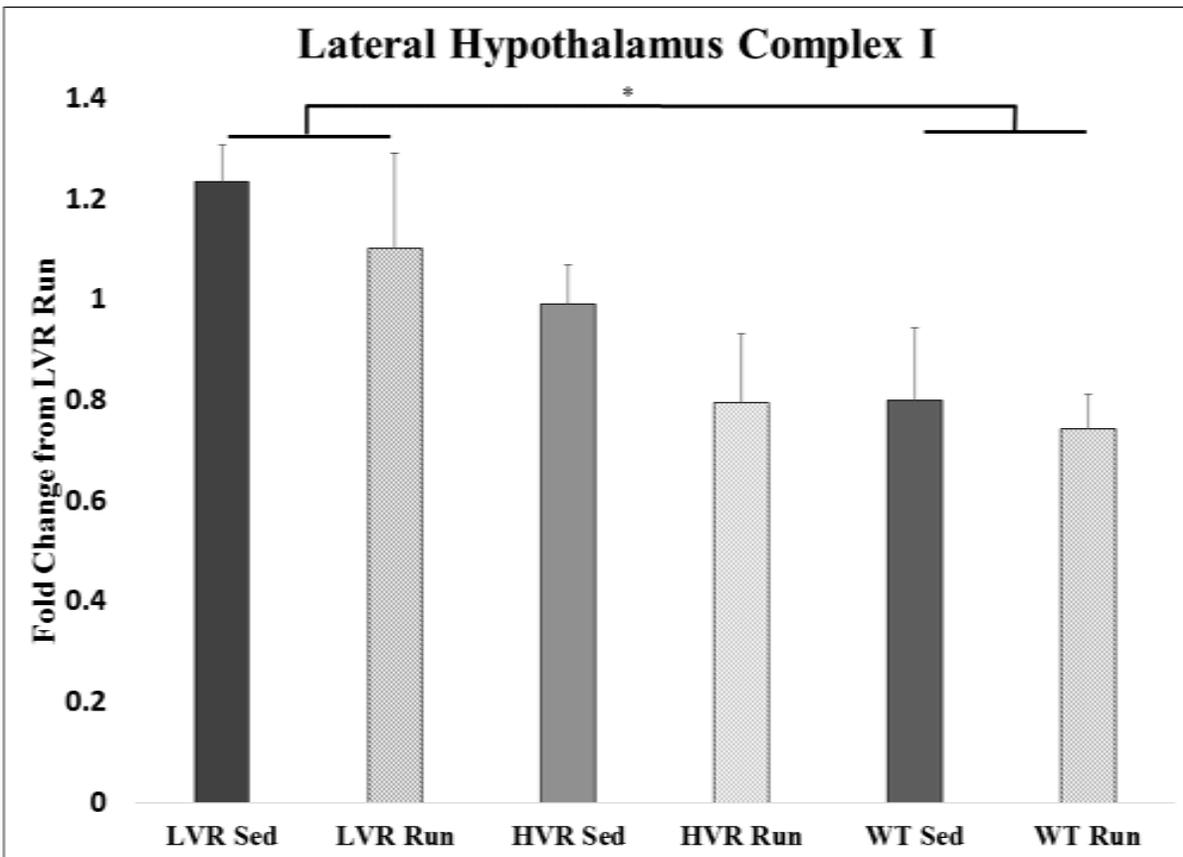


Fig. 21. OXPHOS Complex I Protein Quantification in the LH.

Bars represent the concentration of OXPHOS complex I protein as a fold change, normalized by LVR RUN protein values. The asterisk represents significant differences in the fold change of the protein values. In complex I of the LH, the LVR line presented a 1.51-fold greater level of protein than the WT line ($P=0.11$).

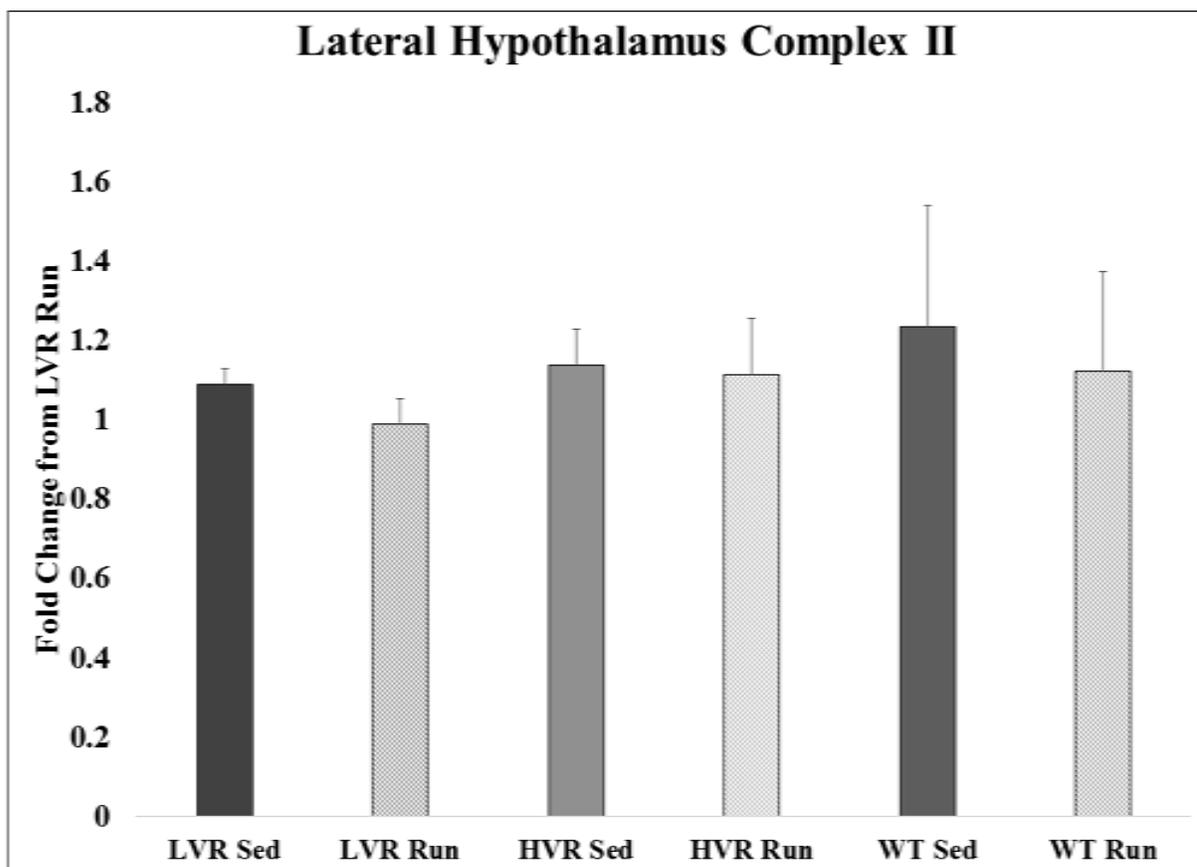


Fig. 22. OXPHOS Complex II Protein Quantification in the LH.

Bars represent the concentration of OXPHOS complex I protein as a fold change, normalized by LVR RUN protein values. In complex II of the LH, there were no significant differences observed.

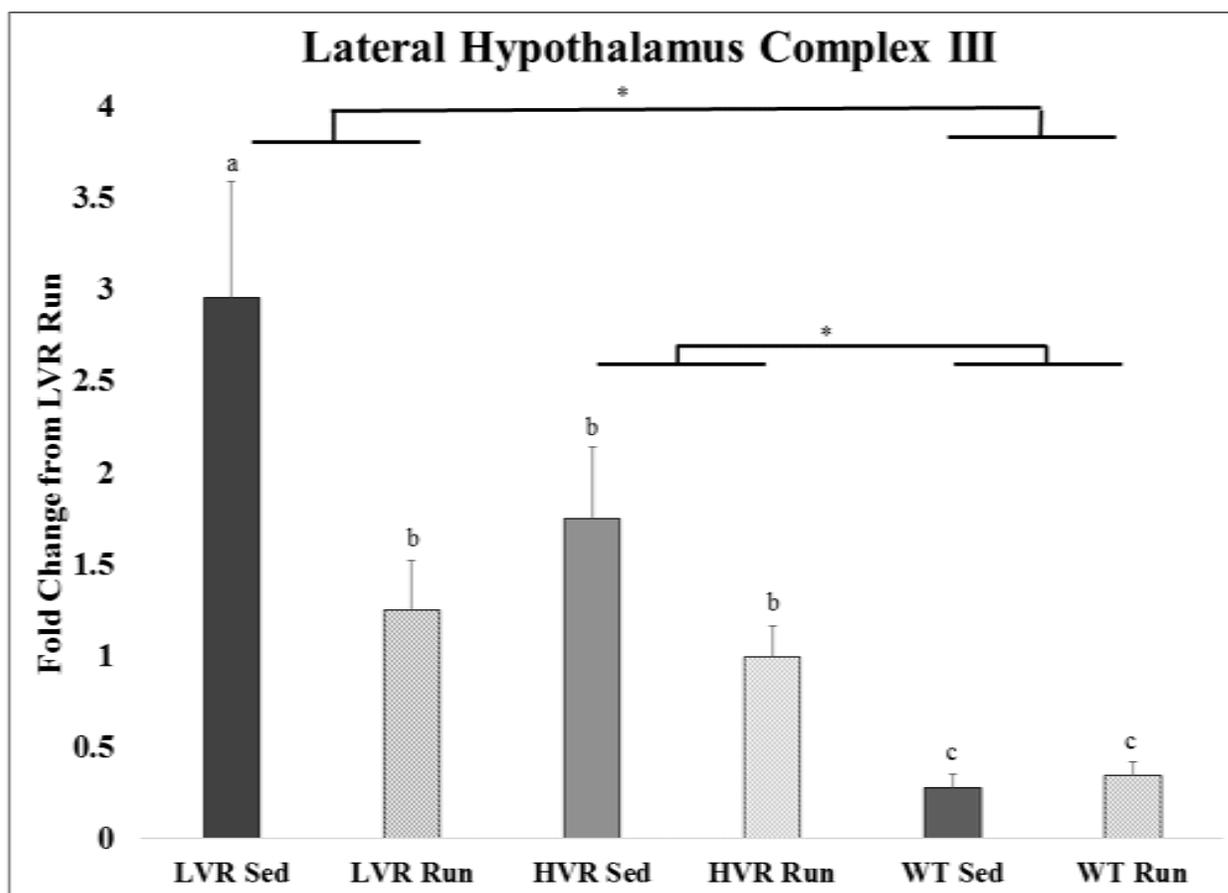


Fig. 23. OXPHOS Complex III Protein Quantification in the LH.

Bars represent the concentration of OXPHOS complex III protein as a fold change, normalized by LVR RUN protein values. The asterisks represent significant differences in the fold change of the protein values. Differences in assigned letters (a-c) also represent significant differences. In complex III, the LVR line was 6.79-fold greater than the WT line ($P = <0.001$). Additionally, the HVR line was 4.43-fold greater than the WT line ($P = <0.001$). Further, the LVR SED group were 10.7 fold greater than the WT SED group ($P = <0.001$) and the HVR SED group was 6.33-fold greater than the WT SED group ($P = <0.001$).

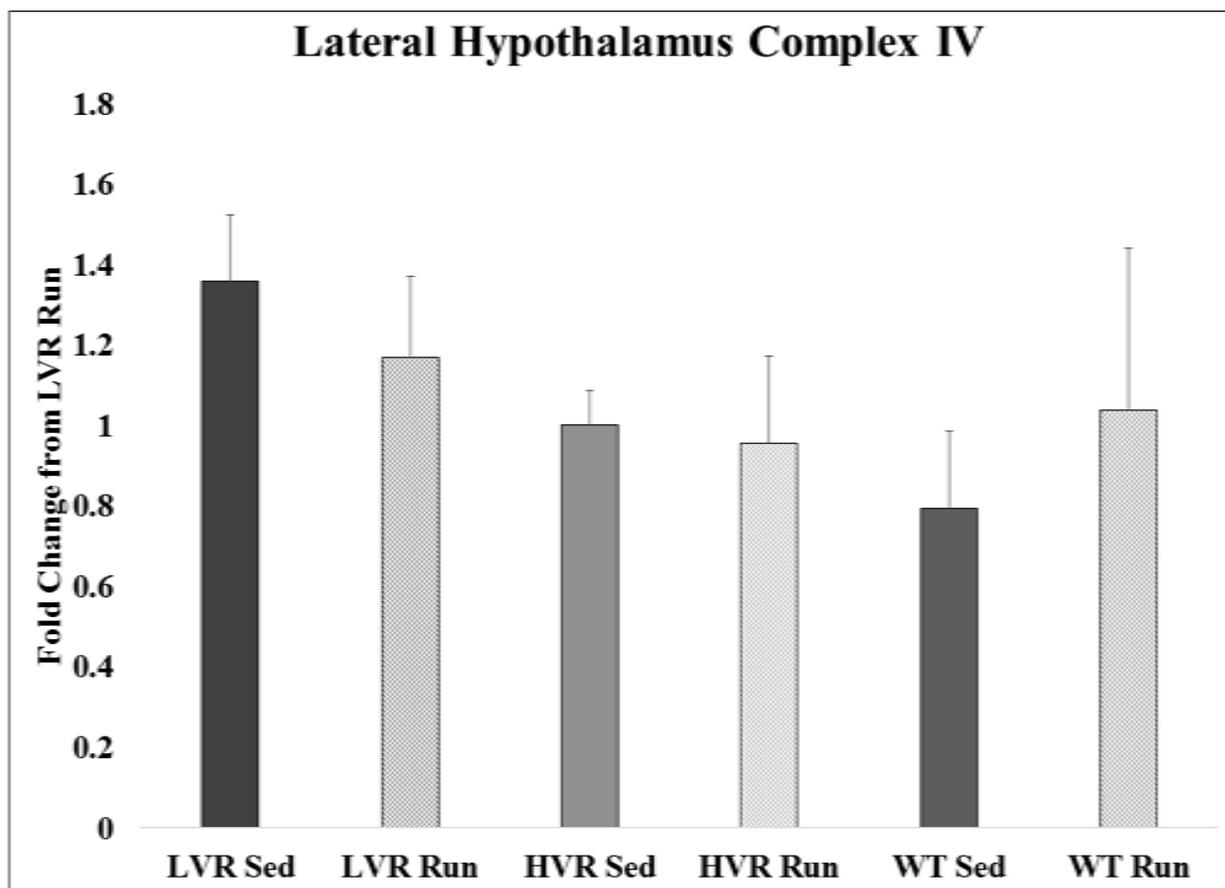


Fig. 24. OXPHOS Complex IV Protein Quantification in the LH.

Bars represent the concentration of OXPHOS complex III protein as a fold change, normalized by LVR RUN protein values. There were no significant changes observed in complex IV proteins.

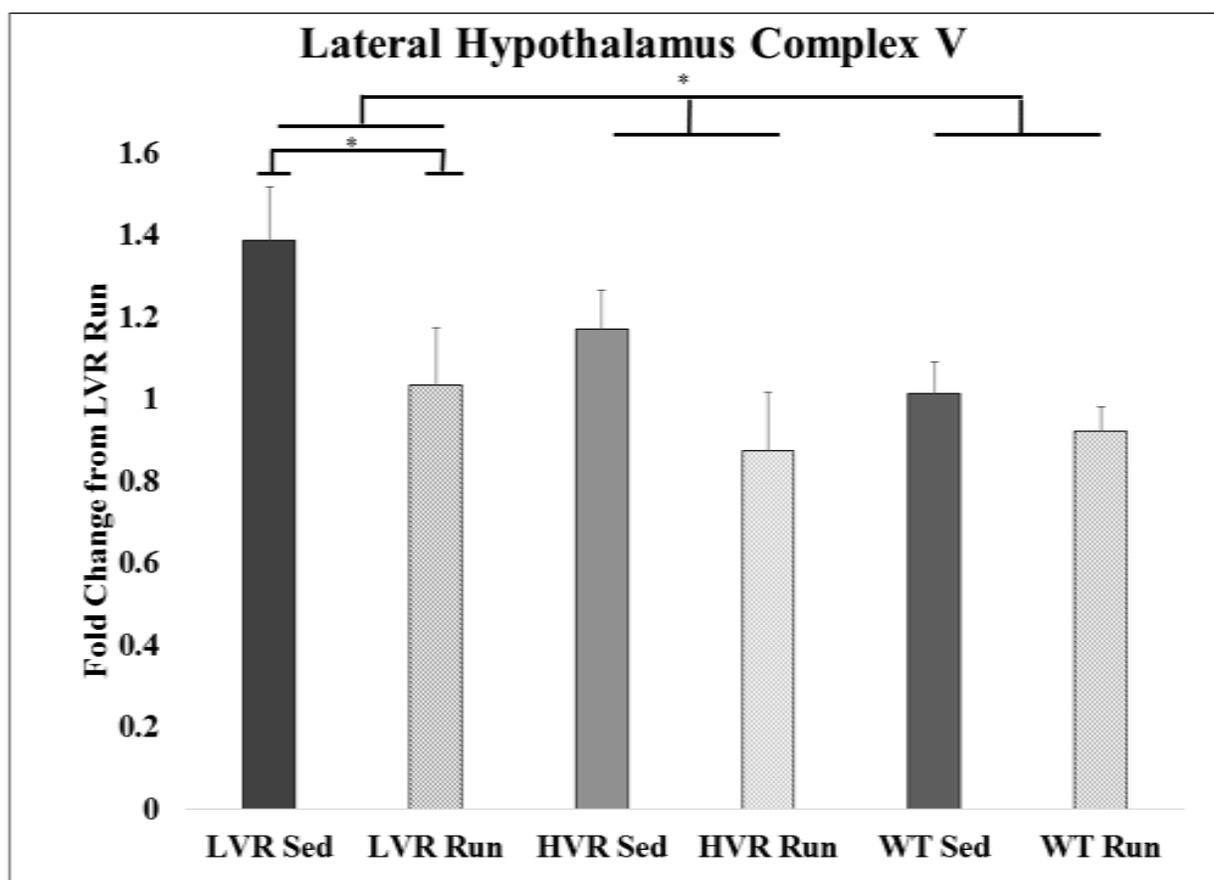


Fig. 25. OXPHOS Complex V Protein Quantification in the LH.

Bars represent the concentration of OXPHOS complex V protein as a fold change, normalized by LVR RUN protein values. The asterisks represent significant differences in the fold change of the protein values. In complex V of the LH, the SED group demonstrated a 1.26-fold greater protein value than the RUN group ($P= 0.011$). Similarly, the LVR SED group was 1.34-fold greater than the LVR RUN ($P= 0.032$).

DISCUSSION

It is a well-known school of thought, that the individual is the product both of their inherited genes, which they receive from their parents, and the environmental factors that have affected those genes throughout the course of their life. Investigators have previously found data suggesting that aerobic exercise increases the biogenesis of mitochondria in several areas of the brain, including the whole hypothalamus and the whole hippocampus (Steiner et al., 2011). Data from our results indicate that the HVR RUN group ran substantially more than the WT RUN and the LVR SED. Building upon this, it was logical to hypothesize that animals with high intrinsic motivation to voluntarily run, such as our HVR line, might have the greatest concentration of mitochondria as well as marker for mitochondrial biogenesis in the NAc, HC, and LH. In support of this hypothesis, it was postulated that the environmental factor of voluntary running would likely increase the amount of mitochondrial concentration in the selected segments of the brain. It is established that only specific portions of the brain increase mitochondrial density in response to forced running on treadmills (Steiner et al., 2011). My hypothesis would suggest that a master regulator of mitochondrial biogenesis, PGC-1 α , would also be higher in HVR than LVR rats. However, the opposite was unexpectedly observed, as described in the next paragraph.

Data from my study consistently supports that the LVR lines have the highest concentration of PGC-1 α , as well as for the highest respiratory chain protein concentrations. Interestingly, one theme that has developed from my study was that the SED animals, primarily among the LVR line, reliably had the largest concentration in

both respiratory complexes and PGC-1 α proteins. Another reoccurring theme was that the HVR line had smaller protein quantities of mitochondrial respiratory complex and PGC-1 α . These findings are difficult to reconcile. Previously, unpublished data in the Booth lab had suggested that the HVR line has a high motivation to voluntarily run; however, HVR PGC-1 α and mitochondrial respiratory complexes did not increase as a result of voluntary running in my current study. These data are interpreted to imply that the NAc, HC, and LH do not likely have an increased major energy utilization role in the motivation to run. Alternatively stated, perhaps mitochondria are not largely involved in the motivation signal to run. It is important to recall that this study did not analyze skeletal muscle. Taken together, I speculate the paradox that a greater energy demand exists in the LVR NAc, HC, and LH when they are SED than during their voluntary running in wheels, which I suggest could be a result of the SED animals' access to social stimulation and perhaps a high level of cage activity.

The NAc is known as a major reward and pleasure hub of the brain. It has previously been suggested that this segment of the brain plays a major role in determining voluntary running behavior in rodents (Knab & Lightfoot, 2010) and that the NAc is related to aspects of motivation for voluntary running (Roberts et al. 2013). The hippocampus is a segment of the brain largely associated with memory processing, mood, and cognition. It has been demonstrated that voluntary-running in mice increases neurogenesis in the hippocampus and is an effective means to attenuate the negative effects of mitochondrial-related diseases such as AD (Tapia-Rojas, Aranguiz, Varela-Nallar, & Inestrosa, 2015). It has also been observed that voluntary-running attenuates memory loss and that exercise increases the size of the hippocampus (Bhattacharya et al., 2015; Varma, Chuang, Harris,

Tan, & Carlson, 2015). The hypothalamus is a segment of the brain that has a role in energy regulation through releasing hormones and regulates body temperature, hunger, thirst, fatigue, sleep, and circadian rhythm. The LH is also involved in the integration and modulation of cardiorespiratory responses during exercise (Barna, Takakura, & Moreira, 2014; Dampney, Horiuchi, & McDowall, 2008). It has also been documented that voluntary-running decreases the reward threshold in the LH, which allows runners to experience an increase in the reward stimuli associated with exercise (Morris, Na, & Johnson, 2012). Thus, it seems paradoxical that the LVR with the least motivation for voluntary running would have a higher mitochondrial concentration in the NAc, HC and LH than the HVR group. I will speculate further on the above in the next paragraphs.

An exception to this trend is that in the NAc, the WT RUN group had a larger increase in complex II proteins than all other groups. One potential explanation for an increase in a single complex could be that it is possible that mitochondrial concentration could play a smaller role in the motivation for voluntary running in the NAc than had been suggested above. Taken together, my data suggests that both those groups innately predisposed to a low motivation to run, such as the LVR group had higher protein content of PGC-1 α and respiratory chain complexes than the HVR and WT, with the aforementioned exception for complex II in the NAc. In further summary for HC and LH tissues, a pattern of LVR or SED animals possessing the highest content of mitochondrial respiratory complexes as well as PGC-1 α continued to be present as compared to HVR and WT.

I have several speculations as to why the previously reported increase in mitochondrial biogenesis did not occur in HVR rats. It is apparent that LVR SED group, as a whole, possessed a higher content of mitochondria and PGC-1 α than their LVR RUN

counterparts. This may be a result of genetic adaptations unintentionally selected for through the primary selection of their unique phenotype of low motivation for voluntary running. It is possible that the LVR line does not require exercise to a larger degree than the HVR in order to maintain their brain mitochondrial health. Contrary to expectations and my initial hypothesis, the selected brain tissue of the HVR rat line did not adapt to voluntary running as highly as the LVR and WT lines in this study. This may also be an unintentional result of selective breeding. Perhaps the HVR rats require a much higher volume of exercise than either WT or LVR lines in order to maintain their brain mitochondrial health. As a result, the HVR rats are much more motivated to run high volumes, because lower levels of running do not confer the advantages that happen in the LVR line.

Strangely, the WT rats also experienced a similar trend of decreased respiratory chain protein content as well as mitochondrial biogenesis in comparison to the LVR line. This may have been the result of multiple factors. It is possible that the measurements were recorded just before adaptations occurred. By this, I mean that the mitochondria have yet to adapt to the oxidative stress that occurs during exercise in the brain. As a result the ROS generated would have diminished the content of mitochondria in the brain. I postulate that if the experiment were to be carried out further that there would have been the “text book” increase in mitochondrial biogenesis as well as an increase in mitochondrial respiratory chain proteins. On the other hand, after 5 weeks, adaptations should have occurred to lessen ROS generation so that ROS-stimulus to mitochondrial biogenesis could be decreased.

Another potential explanation for the LVR having higher mitochondria than the HVR, is that social stimuli confers a larger increase in mitochondrial biogenesis than moderate wheel-running. Early in the course of this study, it was decided to dual-house the SED animals in order to save space, as shelf space on animal rack shelves was limited in the number of cages to be held. As a result, the SED group were given the added benefit of the access to social stimuli, whereas the RUN animals were isolated in their cages because only one rat can be in a cage with a running wheel. It is possible that a social stimuli, particularly in the LVR line, had a great enough impact on the selected segments of the brain to confer advantages in brain health. It is also worth noting that this study occurred very close to the onset of puberty in Wistar rats, which is approximately 40 days of age, and was completed at the end of puberty at approximately 70 days of age. It has been documented that social stimuli have a profound impact in young rats (Serra, Sanna, Mostallino, & Biggio, 2007). It has also been observed in the NAc, that rats allowed social contact display fewer characteristics of mitochondrial dysfunction (Fan, Li, Lichti, & Green, 2013). We have observed that when young rats are separated into isolation with a running-wheel and returned to multiple animal housing they play and wrestle with other rats, which demonstrates the increased cage activity and social interaction that occurs when rats are not isolated.

For my above discussion on time of mitochondrial adaptation in the brain to be explained, I would have to propose that the rates of brain mitochondrial adaptation take longer than skeletal muscle as described next. It has previously been documented that strenuous exercise is required to increase mitochondria content in skeletal muscle (Holloszy, 1967). One can speculate that a continuous running duration over a longer

period of time could have conferred that expected increase in mitochondrial biogenesis in the NAc, HC, and LH. Another possible explanation is that the intermittency of voluntary running is not sufficient to increase mitochondria in the NAc, HC, and LH of the HVR rats. Fitts et al. (Fitts, Booth, Winder, & Holloszy, 1975) data implies that running bouts greater than 10 minutes per day are required to increase skeletal muscle mitochondria during forced running on a treadmill. I speculate that it could take continuous running at greater than 1-2 minute intermittent bouts of voluntary running to increase NAc, HC, and LH mitochondria.

Conclusions

Data from the current study suggests that inherited and environmental factors involved in brain mitochondrial health work through complex systems. The complexity is illustrated by my next conclusions. The current study illustrates important concepts to include: 1) genetic factors such as, motivation to voluntarily run, modulate in brain mitochondrial energetics; and 2) that the effect of social contact in the NAc, HC, and LH may be greater than the effect of short intermittent bouts of voluntary running to increase mitochondrial content. Specifically, SED rats, that are dual-housed with another rat with greater social interaction, possess a higher content of mitochondria in the NAc, HC, and LH than in RUN rats with the largest concentrations of mitochondria occurring in the LVR SED rats.

It is known that exercise is an effective means of preventing brain mitochondrial dysfunction (Cheng, Hou, & Mattson, 2010; M. T. Lin & Beal, 2006; Marques-Aleixo, Oliveira, Moreira, Magalhaes, & Ascensao, 2012; Trushina & McMurray, 2007). The results of this study demonstrate that brain mitochondrial health is a complex and integrative gene-environment interaction. It is apparent that multiple factors should be

considered to maintain or improve one's brain mitochondrial health such as: diet, exercise, and cognitive stimulation. This study highlights the role genes play in the motivation to run as well as their interaction with environmental factors to affect brain mitochondria. Although further research must be done in order to confirm the results of the current study, it is clear that social interaction and exercise are effective means by which to prevent diseases associated with mitochondrial dysfunction.

It is possible that inducing an increase in mitochondrial biogenesis and therefore mitochondrial content in the brain can lead to many positive effects in brain health. Similarly to muscle, it is feasible that increases in mitochondrial content in the brain may improve the brain's endurance and resistance to fatigue when coordinating skeletal muscle during exercise.

There are several potential limitations to this study that can be improved upon in the future: 1) SED animals were dual housed while RUN animals were isolated from social contact; and 2) the time course of the study may have been too short to produce a strong exercise effect in the brain. In the future, it would add support to the findings of the current study to repeat the experimental procedure with several provisions, as described next. It is probable that there could have been an increased content of mitochondria in the RUN group in comparison to the SED if the latter were not dual-housed and the study was carried out for 8-12 weeks, rather than 5 weeks. Additionally, it would be of interest to analyze additional markers of mitochondrial adaptations to exercise such as SOD, SIRT1, and cytochrome c proteins.

PROBLEM SOLVING

Throughout the time course of this project I encountered many obstacles. Despite these limitations and hindrances, I was able to learn a great deal about thinking critically and overcoming difficulties to create tangible solutions to complicated problems. Some of the problems I encountered actually improved my study, as they resulted in a more complete answer to my initial question.

Flaws in Initial Experimental Design

The experimental design initially consisted of only the two selectively bred, LVR and HVR, lines of Wistar rat. However, analysis of the Western blotting data suggested that the LVR SEDs possessed the highest density of mitochondria in the NAc, HC, and LH, which was contrary to the “text book” response to low levels of exercise. After much consideration, I came to several possible conclusions as to why LVR could have more mitochondria. First, it could have been a result of the SED animals being dual-housed, which allowed for increased social stimulation in comparison to those in the RUN group. A second possibility to consider is that the novel line of LVR rats, as a result of selective breeding, were inherently gifted with a higher mitochondrial concentration and a more responsive signaling mechanism to increase mitochondria. Further, the treatment period could have been too short to allow for the full response to running to occur. Previous experiments by Steiner et al., suggested an increase in mitochondria in several brain areas after 12 weeks of treadmill running, whereas our study was 5 weeks. Another study conducted by Aguiar et al. found an increase in hippocampi mitochondrial Complex I activity after 6 weeks of voluntary running; however this study utilized quantitative

reverse transcription-polymerase chain reaction (RT-qPCR) rather than protein content. Although the length of the study may have affected the mitochondrial content in the selected segments of the brain, it is noteworthy that in skeletal muscle tissue 5 weeks of exercise training is sufficient to cause an increase in mitochondrial content. It is possible that voluntary wheel running may take longer because of shorter iterations of running and the slow linear increase in running distance (**Fig. 2a**). In order to verify these data, WT RUN and WT SED groups were added to the study.

Reliability of a PGC-1 α antibody

During the course of the experiment there came to be several learning problems for me with normalizing the Western blotting data between the various membranes and antibodies. Initially to solve this problem, I attempted to normalize to a common sample amongst all blots. As a result of the large number of animals the study, I was only able to include a single common sample per 18 well blot. Unfortunately, on several of the blots there were problems with this sample, because I placed the reference sample in the outside lane of the gel. The outside lane sometimes ran inappropriately. The lesson that I learned was to consult more thoroughly with technical experts before going through with a plan. Therefore, in an attempt to solve this problem, I elected to normalize the blots using a common group (LVR RUN) among all blots. It was also determined that data obtained by the two different PGC-1 α antibodies could not be reliably normalized to allow for an integrated analysis and, therefore, would be analyzed individually.

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