The Beneficial Impact of Exercise on Mechanisms of Neurodegeneration: Potential Therapeutic Approach for Multiple Sclerosis

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by

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AD Alzheimer's Disease, **AP** Action Potential, **ATP** Adenosine Triphosphate, **CNS** Central Nervous System, **DRP1** Dynanim Related Protein 1, **EPO** Erythropoietin, **EPOR** Erythropoietin receptor**, ERRγ** Estrogen Related Receptor Gamma, **H3k4me3** histone H3 trimethyl lysine 4, **Hba** Hemoglobin α, **Hbb** Hemoglobin β, **HIF** Hypoxia Inducible Factor, **KDM5b** Lysine Demethylase 5b, **MS** multiple sclerosis, **NAA** N-acetylaspartate, **NF-κB** nuclear factor kappa-light-chain-enhancer of activated B cells, **NSMASE2** Neutral Sphingomyelinase, **NRF1** Nuclear Respiratory Factor 1, **PD** Parkinson's Disease**, PGC-1α** peroxisome proliferator-activated receptor gamma coactivator 1alpha, **RRMS** relapsing-remitting, **PPMS** primary progressive, **ROS** Reactive Oxygen Species, **SIRT** sirtuin, **SPMS** secondary progressive, **TNFα** Tumor Necrosis Factor alpha

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Chapter 1

Introduction and Background

Multiple sclerosis

Multiple Sclerosis (MS) is a disease of the central nervous system (CNS) characterized by an autoimmune-mediated inflammatory demyelination coupled with chronic neurodegeneration. This a complex disease and there are multiple factors that impact disease progression. Overall, MS results in progressive neurological damage that can be associated with disturbances in motor coordination, pain, insomnia and other sensory-motor phenomena, as well as cognitive changes (Compston 2002; Trapp 2008). The lesions traditionally associated with the symptoms of MS affect the white matter of the brain and spinal cord (Lassman, H. 2018) and for many years, the focus of research on MS centered around these white matter lesions. However, it has been revealed that significant neurodegeneration also occurs in normally appearing white matter of the CNS and throughout the gray matter of the cortex (Trapp 2008). In particular, this process occurs early and persists even during later stages of disease progression where lesion activity has diminished but the neurodegenerative processes are still active (Perez-Cerda, F. et al 2016). This neurologic disease has been identified as a leading cause of non-traumatic chronic neurodegeneration in young adults (Compston 2002), is typically diagnosed between ages of 20-50 years old (Ziemann 2011) and affects more than 2.3 million people worldwide (NMSS).

MS pathology

As stated above, the varied mechanisms associated with MS pathology result in a wide array of symptoms, including fatigue, muscle spasms, tingling and numbness of the face and body, loss of

balance/dizziness along with vision disturbances which make up some of the earliest and most common clinical signs experienced. Importantly, cognitive and psychological problems including depression are also frequent abnormalities that occur (Compston 2002). There are also several well-described subtypes of the disease based on symptomology and pattern of progression (McDonald 2001; Lublin 2014). The most common form, relapsing-remitting MS (RRMS) is associated with periods of clinically identifiable symptoms followed by periods of recovery. Patients are typically diagnosed between the ages of 20-30 years old, however, in many cases these patients progress to a more severe form called secondary progressive (SPMS) where there is a lack of periods of remission. The time for this progression varies greatly between patients but nearly 50% of patients will develop SPMS within 10 years of initial diagnosis. There is also a relatively rare form, primary progressive (PPMS) where symptoms gradually worsen throughout the disease course and there is a lack of periods of remission (Hauser 2006; Bsteh 2016; Perz-Cerda 2016).

While MS involves axonal and neuronal damage it is primarily characterized clinically by an autoimmune component. It is currently appreciated that this response leading to white matter lesion pathology begins with an initial autoimmune insult brought on by antibodies specific for components of the myelin sheath, the multilamellar lipid-protein layer of insulation that surrounds the CNS axons. Myelin is formed by extensions of the plasma membranes of glial cells. Within the brain, oligodendrocytes are responsible for producing the myelin sheath and encasing larger axons (Susuki, K. 2010) (Fig. 1). The sheath is composed of various lipids and proteins that contribute to its insulating properties and structural integrity.

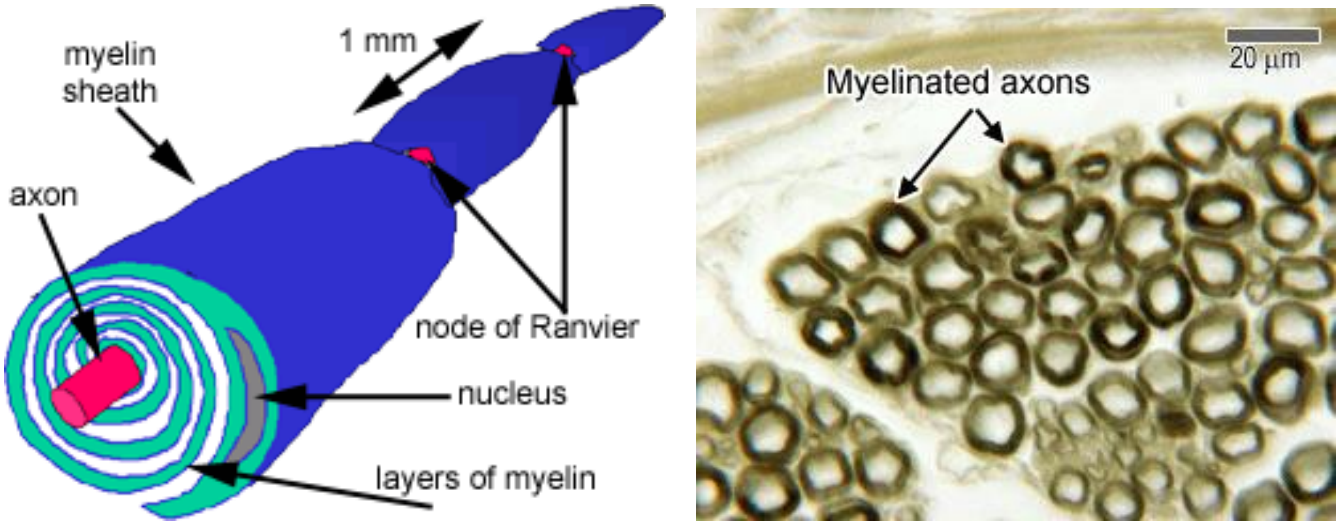


Figure 1. Myelin structure. Left: Illustrates myelin structure as multiple layers of lipid-rich insulating material encasing large axons. Nodes of Ranvier are bare segments of axon that allow for the rapid propagation of action potentials. Right: A cross-section of myelinated axons in the peripheral nervous system. Picture from University of Leeds Histology page.

Unmyelinated areas such as the cell body and nodes of Ranvier that are interspersed along the axon are intended to allow ions to pass in and out so that impulses can be relayed and propagated. The ionic charges that are responsible for the electrical impulses in neurons would be lost in the absence of a sheath in larger axons. In MS, immune cells specific for components of the myelin membrane lead to lesions and ongoing destruction of the sheath. In these lesioned areas, activated immune cells release inflammatory mediators, including products such as reactive oxygen species (ROS), nitric oxide species (RNS) or cytotoxic cytokines (Adamcyk 2016). These inflammatory molecules result in the breakdown of myelin, production of harmful lipid metabolites and cause alterations in cellular metabolism in cortical neurons and their axons (Perez-Cerda et al., 2016). It has also been demonstrated that these immune-mediated mechanisms involve the secretion of cytokines IL-12 and IL-18, both known to be a potent activator of T-cells and both are elevated in MS patients during the progressive stages of the disease (Nicoletti et al., 2010; Cheng et al., 2017). This inflammatory environment has been linked to the progression of axonal injury and demyelination as well (Alboni et al., 2010). Additionally, myelin-specific lymphocytes, including CD4+ T helper 1 (Th1) or T-helper 17 (Th17) cells, have been reported to be activated in the periphery by the interaction with antigen-major histocompatibility complex (MHC) presenting cells and these activated T cells are then able to infiltrate the CNS. Here they secrete additional cytokines such as, IFN-γ or IL-17 leading to demyelination and subsequent axonal damage (Gandhi et al., 2010; Ghasemi et al., 2017; Luckheeram et al., 2012).

Along with damage to the myelin sheath and the white matter lesions of the CNS, there is also early and progressive loss of brain volume which has been linked to the severity of disease

progression and cognitive changes as well (Trapp 2008; Geurts 2008; Fisher 2008). These changes in volume have been suggested to represent the loss of gray matter and supports an involvement of this pathology in disease progression. Various gray matter lesions have been observed and can be classified as type 1, which involve mixed gray and white matter components; type II which are typified by small perivascular areas of demyelination; and type III, which are found at the pial surface and extend into deeper cortical regions (Kutzeinigg 2005; Bo 2003). These lesions are the most common type and are a routinely identified feature in the majority of MS patients (Geurts 2008). It has been proposed that this gray matter pathology underlies the early onset and progression of the cortical atrophy observed in MS and it is a distinct feature from white matter lesions and is also distinct from the normal gray matter atrophy that occurs with aging (Charil 2007). Imaging studies have documented this decrease in cortical thickness in SPMS patients, particularly in frontal, temporal, parietal lobes, as well as in the cingulate gyrus (Charil 2007; Sailer 2003; Calabrese 2007). Further, this decreased thickness can be linked to the progression of disability. Interestingly, in contrast to white matter lesions where inflammatory markers are significantly expressed, the gray matter lesions appear to lack obvious inflammatory activity (Bo 2003; Geurts 2008; Trapp 2008). This lack of observable inflammatory activity may be the result of the limited presence of myelin in these regions and thus immune cells are not necessary for debris removal after demyelination. Regardless, neurodegeneration continues throughout the disease course and understanding the associated mechanisms will be important in driving the development of neuroprotective therapies.

The mechanisms of neurodegeneration are crucial to identify, and in MS as in other neurological diseases, involve interactions between multiple factors. It has been reported that axonal and neuronal degeneration occur early in disease processes and become main contributing factors in brain atrophy over time (Fig. 2). In fact, in later stages of MS where inflammation has subsided, brain atrophy continues and neurodegenerative markers have been noted in non-lesioned areas of MS brain (Lasmann 2010; Confavreux 2006). While inflammatory involvement is certainly an important factor in these neurodegenerative processes, it would also appear that a critical imbalance between axonal energy demand and a limited ability of neurons to provide sufficient energetic support are important factors as well. In fact, this has led to the suggestion that an "inside-out" mechanism may be an initiating factor in the disease. This hypothesis suggests that the neuronal and axonal damage occurs initially due to an as yet identified insult that impairs metabolic processes important for both neuronal energy supply and production of important trophic factors that help maintain myelin health and metabolism. As a result of the impaired metabolism, neurons and myelin components degenerate producing myelin debris that may serve to recruit immune responses in susceptible individuals.

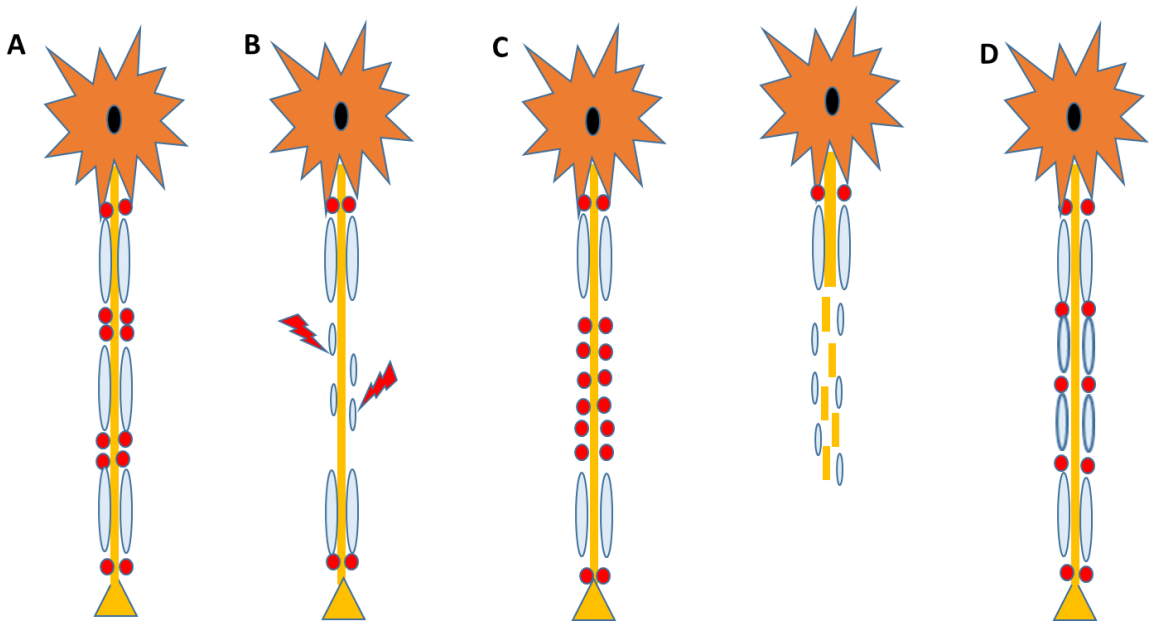


Figure 2. Damage to myelin and neurons in multiple sclerosis. (A) Normal appearing myelinated axon is shown. (B) In MS brains, minor immune-mediated attack (microglial, B-cells) results in axonal demyelination. (C) Most demyelinated axons survive initial demyelination, and neurons recover functions through sodium channel redistribution (C left). Due to high energy demand for signal conduction and loss of trophic support from myelin, demyelinated axons showed slow regression (C right) and eventually degeneration. (D) In certain cases, demyelinated axons can be remyelinated by oligodendrocytes with a shorter myelin sheath along with restoration of a majority of functions (Trapp and Nave 2008). Blue ellipses represent myelin sheath, red circles represent ion channels and pumps.

Understanding the critical nature of neuronal energetics is a crucial concept and it is necessary to describe the metabolic activity required for neurons to produce and propagate the electrical activity that underlies their ability to transmit information throughout the CNS and periphery. Neurons, like all excitable cells, create and maintain a difference in electrical charges across their plasma membranes, termed resting membrane voltage or potential. This uneven distribution of charge is due in large part to the uneven distribution of Na+ and K+ ions on either side of the membrane. When neurons become excited changes in these ion distributions across the membrane create a distinctive pattern of current flow that can jump from node to node in healthy myelinated axons. These electrical events are termed action potentials (AP). During an AP the Na+ and K+ ions move across the membrane along their electrochemical gradients through voltage-gated ion channels and once the event has terminated the neuron needs to restore the ion distributions back to starting conditions in order to "fire" again. In order to maintain and restore the necessary ion distributions, neurons employ tremendous amounts of energy to support the activity of the many ion pumps, in particular the Na+/K+ ATPase. The energy that supports these activities and many other neuronal processes is produced by mitochondria. Importantly, studies in MS and other neurodegenerative diseases have demonstrated that mitochondrial dysfunction is a central feature in disease progression (Dutta 2006; Pandit 2009; Mahad 2009). This disruption in mitochondrial function results not only in diminished ATP production but also in an accumulation of ROS. These factors contribute to a state of cellular distress that can lead to extracellular inflammatory signaling, microglial activation and in some cases, increased permeability of the blood-brain-barrier and infiltration of peripheral immune cells. Within the brain, inflammation begins with activated microglia that

produce TNFa, IL-6, IL-17 and IL-22 (Vallee, A. et al 2018). Activation of pro-inflammatory pathways by these cytokines can then lead to metabolic dysfunction and decreased survival signaling in neurons and oligodendrocytes. All in all, this translates to a loss of, or disruption in normal conduction in neurons initially due to oligodendrocyte death, which is rarely successfully reversed in the disease because of the extremely high energy demand involved in oligodendrocyte turnover. One direct impact of pro-inflammatory signaling is impairment of mitochondrial respiration and production of harmful reactive oxygen species. This metabolic impairment is typical of many neurodegenerative diseases including Alzheimer's disease (AD) and Parkinson's disease (PD). Mitochondrial distress activated pathways in the cell related to cell death and altered metabolism such as the NFkB and HIF-1α pathways also exist.

Factors that influence MS progression

Although the precise etiology of the disease is unknown, it is now generally accepted that neurodegeneration begins in MS before the immunological underpinnings of the disease are present (Lassman 2018), and there is some reason to believe that the early neurodegenerative environment is what determines disease outcomes. The presence of neurodegenerative changes at normally appearing sites within the brain, including gray matter, suggests that other factors give rise to neuronal degeneration, and through release of myelin sheath components in white matter there begins an autoimmune response that exacerbates the disease progression. Interestingly, evidence from progressive MS, which typically shows no active lesions (Lassman 2018) supports neuronal basis for many disease characteristics. In light of disease complexity, it continues to be difficult to ascertain contribution of these two processes in disease onset and

progression. Despite this lack of clear mechanisms, MS provides an opportunity for examining the balance between neuroprotection and neurodegeneration because it exhibits a set of features seen in other neurodegenerative diseases, including aberrant neurotransmitter release, glycolytic changes, increased reactive oxygen signaling, impaired autophagic processes, increased expression of pro-inflammatory cytokines and importantly alterations in mitochondrial morphology and function. As new research emerges, there is more evidence to suggest that mitochondrial function is central to neurodegeneration and thus may also be a target for neuroprotection. Approaches focused on mitochondrial health and function could prove to be capable of affecting disease outcomes. This is due to mitochondria being at the intersection of many pathways related to cell survival, proliferation and inflammation. For this reason, research that focuses on cellular energetics and mitochondrial function is becoming of greater interest in the study of neurodegeneration and as an approach to preventative therapies.

Mitochondrial history and physiology

Mitochondria are the organelles within the cell responsible for producing most of the cellular energy. The mitochondrion consists of an inner and outer membrane layer. The outer membrane serves to protect the organelle and regulate passage of materials in and out, such as water and molecules that are tagged for transport to the mitochondria. The inner membrane is convoluted into folds known as “cristae” that house many of the enzymes required for energy production, especially the electron transport complexes.

Mitochondria arose within the cell recently in evolutionary history through endosymbiosis, or the internalization of mitochondria by cells roughly 1.4 million years ago (Martin & Mentel

2010). Prior to this event, cells relied on the splitting of glucose, or glycolysis, within the cytoplasm and regeneration of electron acceptors using fermentation to produce energy. The emergence of mitochondria within the cell led to a much more efficient yield of energy. Mitochondria accomplish this by housing the enzymes responsible for the tricarboxylic acid cycle and electron transport chain—the pathways responsible for taking the products of glycolysis and breakdown of fat and proteins from the cytoplasm and further reducing them for a larger net capture of energy in the form of the storage molecule known as ATP (adenosine triphosphate). Typically, the sum of these three pathways, commonly known as aerobic respiration or oxidative phosphorylation, will produce 36 molecules of ATP to glucose molecule to use in energy-requiring processes within the cell. For this process to occur, cells require molecular oxygen to serve as the final electron acceptor at the end of the electron transport chain. Cells with high energy demands require a high number of mitochondria to meet their energy needs. Neurons in particular rely on localized enrichment of mitochondria due to their high demand for energy. This immense metabolic demand is due to the highly differentiated nature of neurons and their need for large amounts of ATP that is necessary for maintenance of ionic gradients across the cell membranes, for neurite outgrowth, axon elongation, action potential generation and for neurotransmission (Suzuki, Hotta, Oka 2018). Since most neuronal ATP is generated by oxidative metabolism, neurons critically depend on mitochondrial function and oxygen supply

Over the past few decades, the importance of mitochondrial dynamics in neurological health has become more evident. Unlike other cellular organelles, some of the mitochondrial proteins are encoded for by their own genome, a circular piece of DNA about 16.5kB in size (Keough &

Chinnery 2015). This circular DNA is a remnant of the ancient bacterial origin of mitochondria. Like nuclear DNA, mitochondrial DNA undergoes replication during mitotic division throughout the lifespan of an individual, making it susceptible to DNA changes characteristic of the aging process. Additionally, mitochondria continue to undergo replication and division in post-mitotic tissues, including neurons, highlighting them as major players in the aging of neurons and muscle cells. Most mitochondrial dysfunction arises from changes in the respiratory complex proteins of the electron transport chain, which is central to the decline of oxidative phosphorylation seen in aging. It is important to note here that in addition to mitochondrial-encoded proteins, most of the crucial components of the electron transport complexes are encoded by genes in the cell nucleus. Because mitochondria stand at a junction between energy metabolism and cell survival, they also moderate the efficiency of several other processes that are critical to the cell such as lipid biosynthesis, calcium signaling and apoptosis—all processes that are aberrantly regulated in neurodegenerative diseases.

Mitochondria and inflammation

Mitochondria are a source of ROS within the cell. ROS occur naturally in organelles with high oxygen consumption. In mitochondria, the electron transport chain complexes I & III produce superoxide as a byproduct of respiration. The complexes reduce an additional complex known as coenzyme Q, which transfers additional electrons to molecular oxygen forming the extremely reactive anion known as superoxide (Phaniendra, A. et al. 2015). The enzyme superoxide dismutase is responsible for converting superoxide into hydrogen peroxide, which

can then be broken down into catalase. However, not all superoxide and hydrogen peroxide can be removed through this process. In situations where metabolic rate is extremely high, such as during early stages of inflammation, they can accumulate. These species are harmful to the cell and can elicit cellular damage and even death as a result of cytochrome C release and activation of its associated apoptotic pathways that involve various caspase enzymes. The production of ROS is both cyclical and paradoxical. ROS are produced in response to cellular distress, and cellular distress can produce ROS. Paradoxically, an excess of ROS can be seen in hypoxic conditions as a means of signaling distress within the cell (M. Murphy 2009). ROS increases can also lead to cytokine release and a progressive state of inflammation within the cell. These changes in the inflammatory milieu may further be reflected in the alterations in the production of various lipid metabolites, including ceramides which are derived from myelin lipid metabolism. The lipid known as sphingomyelin is a major component of membranes in cells types of the brain, especially oligodendrocyte cells—the glial cell type responsible for axon-insulating myelin (Halmer R, Walter S, Faßbender K. 2014). Sphingomyelin is often found deficient in MS brains at the sites of lesions and inflammation (Fyrst H 2010). In this inflammatory environment, it has been shown that activation of the TNFα1 receptor stimulates neutral-membrane associated sphingomyelinase 2, which is the enzyme responsible for the degradation of sphingomyelin to a class of lipids known as ceramides (Martinez, T.N. et al. 2012). Ceramide lipid levels are of great relevance to neuroinflammation (de Wit. Et al. 2019), and can initiate activation of caspase cascades which then cause destruction of mitochondria (Sawada M, 2001). These lipids have various individual effects based on their fatty-acid chain length. Ceramide C6 increases pro-apoptotic caspase 2, C16 aids in channel formation in the

mitochondrial outer membrane and C18 inhibits cytochrome C activity (Qin J 2012, Groves A 2013). Formation of ceramides and activation of other second messenger systems increases ROS in mitochondria impairing metabolism. Ceramide production by astrocytes is now thought to be a biomarker of several neurodegenerative diseases characterized by neuroinflammation, including AD, PD and frontotemporal lobar dementia (de Wit et al. 2019). Additionally, this pathway is important in inflammatory states seen commonly in obese individuals.

Mitochondria in neurodegeneration

Evidence supports that mitochondrial dysfunction plays an important role in neurodegenerative diseases and impacts additional cellular pathways in the brain (Mao 2009; Su 2013). In fact, in our earlier studies we have observed that mitochondrial function is compromised in neurons as a result of altered expression of nuclear-encoded genes necessary for mitochondrial respiration (Dutta 2006; Dutta 2007; Dutta 2012). In addition, it has been noted using proteomic analysis of cortical mitochondria that mitochondrial proteins involved in respiration are differentially altered in MS brain (Broadwater 2007). Further, altered levels of hemoglobin β (Hbb) were also associated with changes in neuronal energetics and differences were pronounced in MS cortex when compared to controls (Broadwater 2007; Sadeghian 2016). Further evidence includes findings from studies using in vivo magnetic resonance spectroscopy (MRS) showing that an important marker of neuronal mitochondrial function, N-acetylaspartate (NAA), was reduced in neurodegenerative diseases (Ge 2004; Cader 2007; Schuff 2006) (Fig. 3). Interestingly, NAA is produced almost exclusively by neuronal mitochondria by the enzyme N-acetyltransferase and is one of the most concentrated molecules in the CNS reaching levels of up to 10mM (Pan and

Takahashi 2005). Reductions in NAA have been observed in various neurological diseases and appears to precede brain atrophy which has been used to imply that dysfunction of mitochondria may occur prior to and be a contributing factor in neurodegeneration. Thus, this recognized involvement of mitochondrial health and metabolism in neurodegenerative processes supports studies aimed at understanding mechanisms that control mitochondrial function.

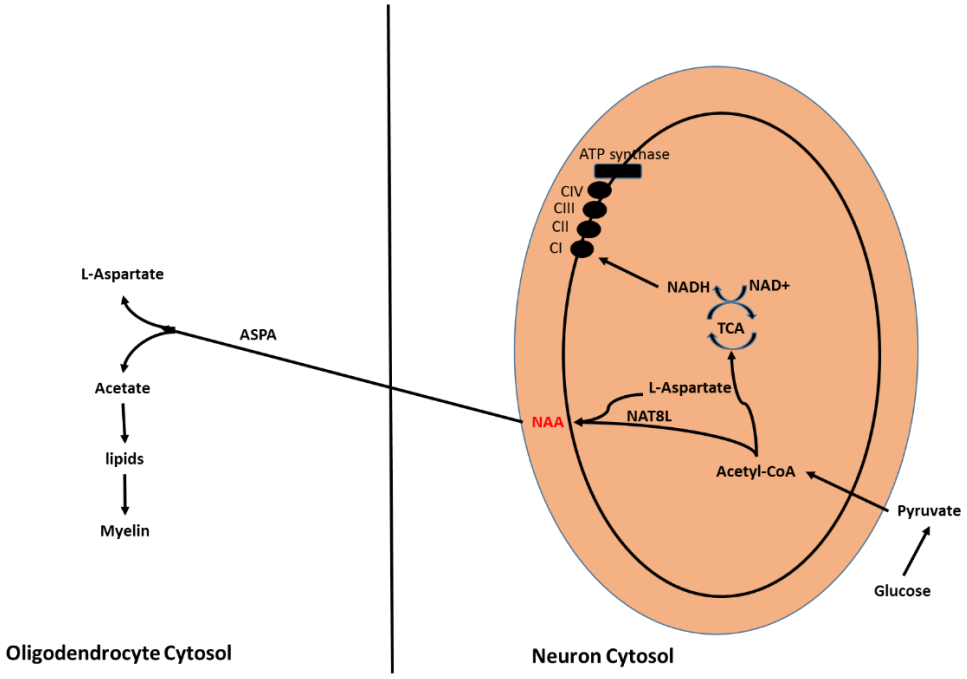


Figure 3. NAA metabolic pathway. Schematic depicting NAA metabolic pathways and their relationship to energy metabolism pathways. Diagram depicts the reactions involved in NAA synthesis from L-aspartate and acetyl-CoA in neuronal mitochondria and subsequent catabolism by ASPA to aspartate and acetate in the cytosol of oligodendrocytes. NAA N-acetyl aspartate, ASPA aspartoacylase, NAT8L (N acetylaspartate sythetase).

Hemoglobin in neurons

The hemoglobin beta (Hbb) peptide is a subunit of the blood oxygen carrying protein known as hemoglobin. Erythrocyte cells produce and carry hemoglobin in abundance, but hemoglobin subunits have also recently been discovered in many other cell types, including those of the brain (Biagoli, 2009). Its presence in these other tissues suggests that it may possess additional roles to its well-studied role in O2/CO2 transport (Nishi 2008; Rahaman 2013). In previous studies we have also identified the presence and extent of hemoglobin expression in the rat brain. In these studies, we observed that hemoglobin expression was localized throughout the brain and can also be identified in cultured neurons (Fig 4). Further, we confirmed that its expression was regulated by oxygen concentration and in overexpression studies, hemoglobin was able to increase neuronal respiration through interactions with mitochondria that included increased expression of mitochondrial genes. This latter effect was also associated with increases in histone H3K4 trimethylation (Li et al 2015). Additional factors shown to regulate its expression in neurons include rotenone, hydrogen peroxide and excess NO (Li et al. 2015).

Hemoglobin in neurons and potential roles

Hemoglobin alpha and beta subunits have remarkably wide distribution throughout the brain. In fact, both are expressed in neurons of the striatum, substantia nigra, hippocampus and cortex in both rat and human brains (Richter, et al 2009). Transcript levels of each respective subunit can vary by region, but functionally their roles are similar with hemoglobins serving as regulators of oxygen levels and respiration within neurons. However, it has also been demonstrated that hemoglobin α (Hba) and β (Hbb) subunits can carry out diverse physiological

functions in many different cell types. β-globin contains five functional loci (ε-GΥ-AΥ-δ-β) that transition their expression during development to maturation in humans, such as, fetal δ-globin to adult β-globin expressed in red blood cells (Kingsley et al., 2005).

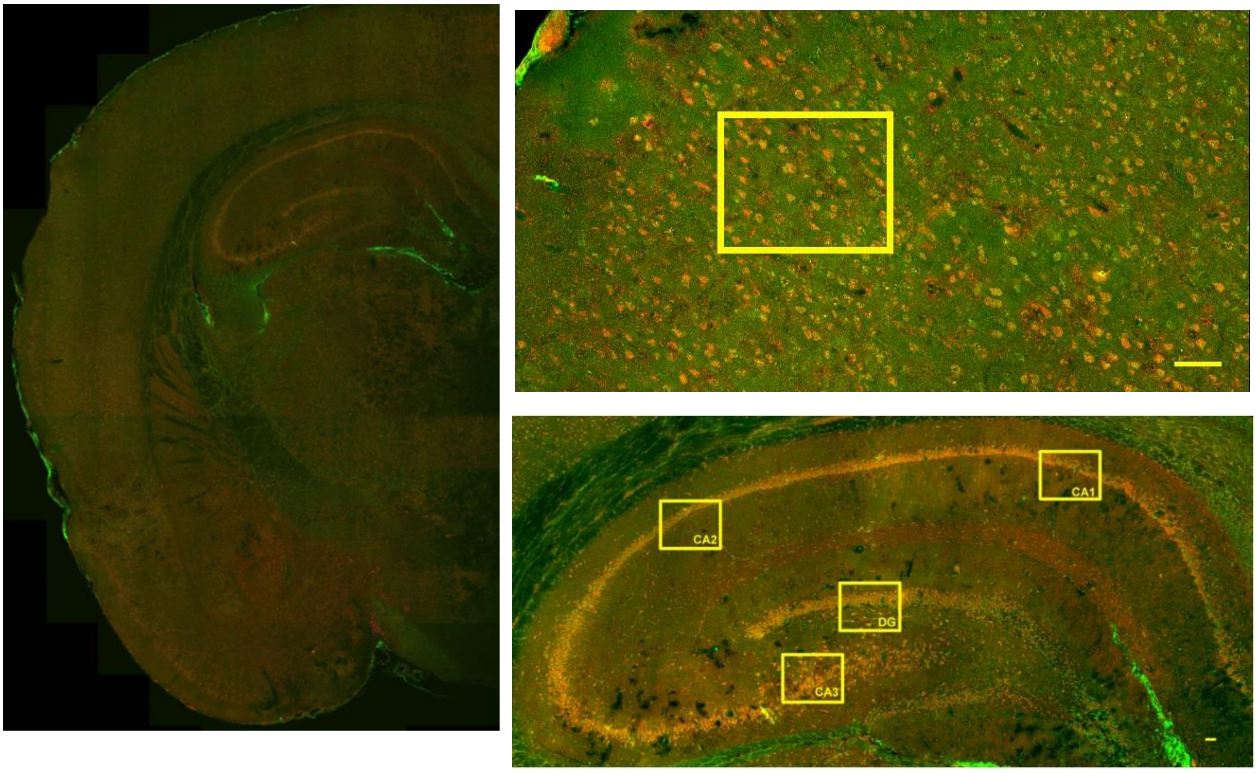


Fig 4. Hemoglobin expression in rat brain. (A) Overview of adult rat brain sections stained with anti-hemoglobin α and anti-hemoglobin β. Hemoglobin is expressed in neurons in rat cortex and hippocampus. Hemoglobin α and hemoglobin β colocalize in the cerebral cortex and hippocampus. Images were taken at a magnification of 10X. Representative hemoglobin α and hemoglobin β expression in the (A) whole slice, (B) cortex, (C) hippocampus rat brain sections. Scale bars represent 100 microns. Hemoglobin is mainly localized at the cell body and processes of neurons. Scale bar represents 100 microns unless labeled otherwise. Unpublished data Li and Freeman 2015.

The hemoglobin β subunit, is expressed without the α subunit in macrophages assisting in scavenging nitric oxide (Liu et al., 1999). In endothelial cells, Hba regulates NO availability and regulates vascular tone in response to O_2 concentrations (Straub et al. 2012). Importantly, as documented above, hemoglobin has been shown to be present in neurons of the human and rodent brain (Avivi et al., 2009; Ritcher et al., 2009). It has been suggested that the expression of hemoglobin in neurons may enhance oxygenation and support mitochondrial respiration under oxidative conditions (He et al., 2009).

Changes in neuronal Hbb expression or subcellular localization have been linked to MS, AD, and PD (Brown et al., 2016; Ferrer et al., 2011; Freed and Chakrabarti, 2016). Elevated levels of HBA2 in red blood cells in peripheral blood have been correlated with a less severe course of MS in a study observing patients experiencing the progressive disease stage suggesting a neuroprotective role (Ozcan et al., 2016). Further, we have previously reported that Hbb levels are increased in mitochondrial fractions obtained from gray matter of MS postmortem brains when compared to controls (Broadwater et al., 2011). This could suggest a compensatory protective mechanism, since we also noted that treatment with erythropoietin (EPO) was linked to increase neuronal Hbb and provided protection in the cuprizone mouse model of MS (Singhal et al., 2018). Altered expression of hemoglobin chains have also been reported in Huntington Disease (HD) and it was reported that huntingtin (Htt) protein interacts with Hbb in the brain (Kaltenbach et al., 2007). Additionally, in this study Hbb was assigned to the cellular metabolism functional category as determined using gene ontology (GO) analysis. In AD, hemoglobin levels were found to be decreased in postmortem cortical samples (Ferrer et al., 2011), while another study reported that hemoglobin interacts with amyloid-β supporting a role in AD pathology

(Chuang et al. 2012). More specifically, it seems that amyloid- β aggregates as a means of neutralizing free hemoglobin following permeabilization of microvasculature in the brain (Chuang FY. Et al 2012). These leakages can occur because of brain trauma, severe inflammation or as part of the aging process. Free hemoglobin in the brain is highly toxic, so it is likely that aggregates are meant to serve as protection. This contrasting relationship between intracellular and extracellular hemoglobin in AD has been proposed as evidence that intracellular hemoglobin serves a separate role from blood hemoglobin, with intracellular hemoglobin more likely to serve as an oxygen sensing mechanism that can initiate appropriate compensatory responses within the cell rather than serving solely as an oxygen delivering molecule (Altinoz, M. 2019).

In Parkinson's disease changes in mitochondrial localization of hemoglobin was correlated with disease duration (Shephard et al., 2014). It has been proposed that the increased production of hemoglobin in PD demonstrates a role for hemoglobins as energy sensors that could potentially increase or decrease the cellular metabolism during states of cellular distress. These studies provide evidence to support the suggestion that changes in hemoglobin expression and localization are involved in the neurodegenerative processes and suggest that understanding hemoglobin signaling in neurons may provide new therapeutic targets.

Epigenetic mechanisms in neurons

Epigenetic alterations to histones have also been linked to neurodegenerative processes. Histones are proteins that comprise the core structure of a nucleosome. Nucleosomes are

composed of 147 base pairs of DNA wrapped around histone core proteins H2A, H2B, H3, and H4 (Marino-Ramirez et al., 2005; Tremethick, 2007). Histone tails on nucleosomes are abundant in lysine and arginine residues, and these residues are sites for modifications in response to extracellular signals (Huynh and Casaccia, 2013). Histones are proteins that are involved with the packaging of DNA into chromatin. The degree of tightness to which DNA binds the histones impacts the expression level of genes. In this scenario, more tightly bound DNA leads to lower expression, and more loosely bound DNA leads to higher expression. The most common form of nucleosome modifications includes the addition of amino acids to the histone tails including acetyl groups, methylation, citrullination, sumoylation, and ubiquitinylation (Hyun et al., 2017). The addition of acetyl groups to histones is catalyzed by histone acetyltransferases (HATs) and deacetylation is catalyzed by histone deacetylases (HDACs) (Huynh and Casaccia, 2013). Histone methyltransferases (HMTs) are responsible for methylation of lysines and arginines on histone tails. Histone demethylases (KDMs) that demethylate these residues on histones have also been reported (KDMs) (He et al., 2018). The activity of these enzymes determines the nature and extent of the histone post-translational modifications present in cells. These epigenetic alterations also create patterns within the chromatin that play an important role in regulating transcription in the modified regions (Marino-Ramirez et al., 2005; van Leeuwen and Steensel, 2005). Actively transcribed regions of chromatin (euchromatin) have been linked with high levels of lysine acetylation of K16 on H4 at promoter regions. In addition, the histone H3 has various methylation patterns that influence transcription. One particular modification, the trimethylation of histone H3 on lysine 4 (H3K4me3) methylation pattern, is associated with DNA transcriptional activation (Kouzarides, 2007; Taylor et al., 2013). Transcriptionally

repressed regions (heterochromatin) are associated with low levels of lysine acetylation or trimethylation of lysine at positions K9, and K27 on histone H3, as well as monomethylation of lysine K20 on histone H4 (Saksouk et al., 2015; Huynh and Casaccia, 2013). Acetylation of histone tails causes changes in the nucleosome regarding its structure and folding that enable transcriptional factors to access DNA for transcription (Javaid and Choi, 2015).

Histone methylation has been found to be altered in neurological disease states (Singhal et al., 2015; Codrich et al., 2017; Webb and Guerau-de-Arellano, 2017). Methylation occurs by the addition of a methyl group on arginine or lysine residues on histone tails. The methyl groups are donated from S-adenosyl methionine (SAM) to the HMTs that then methylate histones (Loenen, 2006). The transcriptional impact of histone methylation depends on the number of methyl groups and the amino acids to which they are added. The methyl groups are recognized by proteins with methyl binding domains including, plant homeodomain (PHD) fingers and (tryptophan-aspartic acid) WD40 motif repeats (Greer and Shi, 2012). Most histone methylation leads to silencing or repression of gene expression. However, trimethylation of histone H3 on lysine4 (H3K4me3) activates transcription (Barski et al., 2007). Histone modifications have been shown to be altered in postmortem cortical tissue in MS brains, as well as in AD and PD brains (Wen et al. 2016). We have also reported that H3K4me3 was significantly decreased in the gray matter of diseased brains from MS patients compared to controls (Singhal et al., 2015). Importantly, in the same study, methyl donors of the methionine metabolism cycle were also found to be significantly decreased resulting in a reduction in H3K4 trimethylation. Studies have also suggested that inhibitors of histone acetylation or demethylation may impact neurologic diseases (Zhang et al., 2018). For example, in dendritic

cells, inflammatory gene expression is regulated by relative levels of the active H3K4me3 methyl mark compared to the repressive H3K27me3 (Donas et al., 2016; He et al., 2018). Increased levels of H3K4me are also associated with improved outcomes in some cancers (Cox et al, 2019). In addition to the levels of H3K4me3, their placement along genes follows a particular pattern in healthy individuals, and this pattern may be altered when a person is unhealthy, as meta-analyses of malnourished children have found (Uchiyama et al. 2018). Patterns of enrichment in H3K4me3 have also been found to be of potential importance in anxiety disorders and other related health outcomes (Cittaro et al. 2016). Changes in H3K4me3 levels and distribution are a natural part of the aging process as well. As seen with pathological conditions, the direction of the change varies widely and the outcome has a stronger relationship to the genes where it is found than the overall levels. A study using Wistar rats examined the relationship between histone marks, aging and exercise, and it was found that H3K4me3 changed in the location of genes associated with synaptic plasticity, memory and neuronal activity (Ferreira de Meireles et al 2019). The type of exercise also influenced the degree of change seen. These effects were not present in younger rats at the loci examined, but little other research has looked at H3K4me3 changes in exercised animals. Because our lab has previously demonstrated a relationship between H3K4me3 and mitochondrial health in the MS brain, we are interested in better defining the relationship between exercise and this histone marker.

Epigenetics and oxidative conditions

MS is a complex and multifactorial disease and there is significant evidence suggesting that environmental factors impact disease susceptibility, as well as progression. This environmental

influence appears to occur via the epigenetic regulation of gene expression that occurs through modifications to chromatin structure as opposed to changes in DNA sequences. Many studies have suggested a role for epigenetics in MS as a result of reports detailing modifications to chromatin under conditions of stress, including hypoxia, oxidative stress, and mitochondrial dysfunction (Robertson et al., 2014; Johnson et al. 2008; Kreuz and Fischle, 2016; Codrich et al., 2009). These environments that have been noted in MS diseased brain may cause alterations in chromatin structure through mechanisms that include inhibition of histone demethylases that results in increased levels of methylation, particularly H3K4me3. Of significance, it has been reported that histone demethylases of the KDM family belong to the Fe^{2+}- and 2-OG-dependent oxygenase super family which have been shown to be inhibited by hypoxia. The resulting alteration in histone methylation patterns has been reported to result in transcriptional repression (Thienpont et al., 2016; Hatch et al., 2017; Chakraborty et al., 2019). Further, KDMs- O_2 dependence in hypoxic tissues supports the possibility that their activity is impacted by the availability of O_2 (Hancock et al., 2017) (Fig. 5). The enzyme KDM5B activity results in the removal of methyl groups from lysine 4 of histone H3 (H3K4me3). Thus, studies on the O_2 dependence of KDMs in hypoxic tissues supports the possibility that their activity is modulated by the availability of O_2 (Hancock et al., 2017). Hemoglobin contains a heme-prosthetic group (Fe-protoporphyrin IX) which binds to O_2 and NO and has been reported to upregulate H3K4me3 levels suggesting the possible interaction of hemoglobin and KDMs in relation to O_2 binding and KDM activity. Alternatively, oxidative environments may exert effects on HMTs as a result of the reduction in the availability of the methyl donor SAM under these

conditions. The resultant effect would be a reduction in HMT activity leading to histone H3K4me3 hypomethylation (Kreuz and Fischle, 2016).

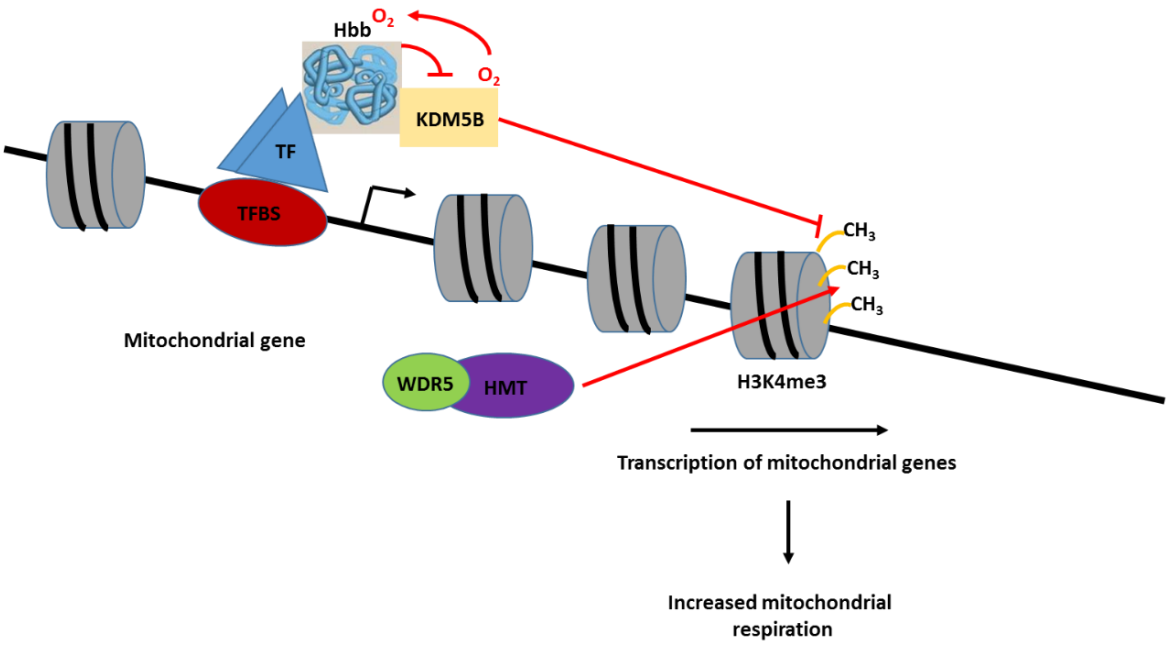


Figure 5. Hbb signaling in neurons. We have found that Hbb inhibits the KDM5B histone demethylase by sequestering away O_2 necessary for its activity, leading to increased methylation of H3K4 to H3K4me3 by the Set histone methyltransferase (HMT). WDR5 is a component of the HMT that methylates H3K4 to H3K4me3. We have previously published that Hbb-mediated increases in H3K4me3 activates gene expression. TF; transcription factor, Methyl group,-CH3 groups. Adapted from Gibson and Kraus, 2011, Mol. Cell, 41, 497-499.

Exercise and the brain

Exercise has long been known to protect against cardiovascular diseases, as well as, reduce the risks of several cancers and metabolic disorders (Blair et al. 1999). For instance, in models of Type 1 Diabetes, aerobic exercise has been shown to decrease plasma levels of the pro-inflammatory cytokines TNFα and IL-6 (Kazemi. 2019). In the central nervous system, exercise has been linked to significant benefits related to neurodegenerative processes, improvements in depression (Alkhadi. 2017) and cognitive function (Ahmed et al. 2017; Stranahan et al. 2012). Exercise has been suggested to potentially be a disease-modifying approach to the treatment of cognitive dysfunction in AD (Frederiksen Et al. 2018; Law at 2018), as well as a way to ameliorate the symptoms of both MS and PD (Doring et al. 2012; Frazzitta et al. 2013; Schenkman et al. 2018). Some of the benefits of exercise for the brain have been linked to structural and molecular changes. Increases in brain derived neurotrophic factor, increased serotonin availability and increased epinephrine have all been identified as ways that exercise can alleviate depression and improve cognition (Gujral et al. 2017). Meta-analyses that have looked at the effect of exercise on depression and anxiety have found that most types of physical activity are capable of reducing symptoms (Kvam et al. 2016: Carek et al. 2011). It has been proposed that the benefits of exercise for the brain occur primarily through increased expression of BDNF, EPO, as well as other molecular and structural changes in regions including the hippocampus, prefrontal cortex and amygdala (Gujral et al. 2017) (Fig. 6). Exercise may also increase the availability of transmitters (serotonin, norepinephrine) and alter activity of the hypothalamic-adrenal-pituitary axis in addition to reducing inflammatory factors—all actions

that can benefit the depressed brain, as well as abrogate the course of many neurodegenerative diseases.

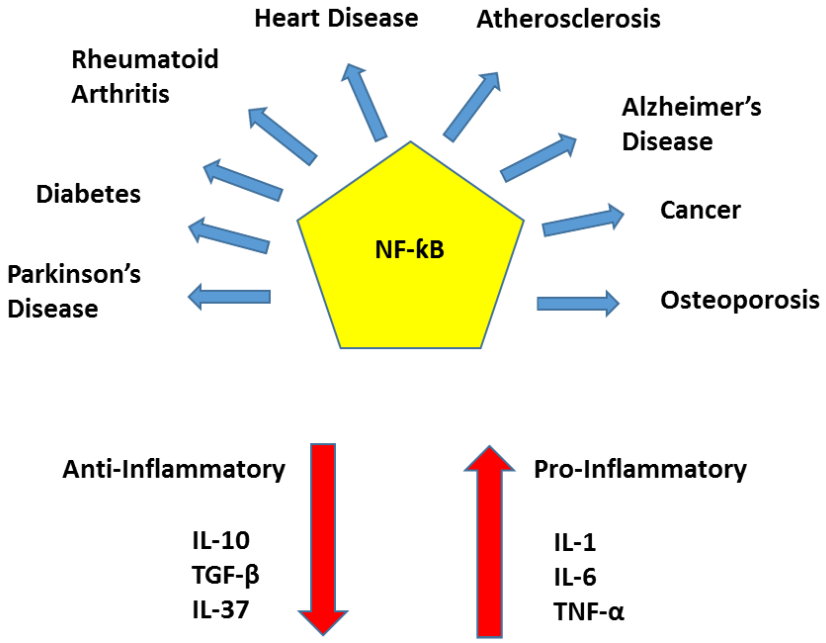


Figure 6. Inflammatory pathways impacted by exercise. The relationship between the pro-inflammatory milieu and diseases of aging. It is generally accepted that regular exercise switches the milieu from pro- to anti-inflammatory, helping to reduce risk of disease, and in some cases disease progression (I.M. Rea et al 2018).

However, there may be additional players in the brain that help reduce neuroinflammation and promote positive structural changes. The relationship between exercise and cognitive function is still being elucidated (Ahmed et al. 2017; Stranahan et al. 2012). In AD, exercise has been suggested to potentially be a disease-modifying approach to the treatment of cognitive dysfunction (Frederiksen et al. 2018; Law at 2018). Studies on these benefits have revealed that

exercise can in some cases increase cortical thickness in AD patients, but they have yet to explain the precise mechanisms for the cognitive improvements that can occur in the absence of structural changes (Frederiksen et al. 2018). It is possible that some of the biochemical changes in AD allude to the reason why exercise improves cognition. Studies on AD and other types of dementia have shown decreases in important biological molecules such as hemoglobin (Koyama et al 2016), and the physiological impact of exercise may be capable of reversing these effects. In PD, when used as an adjunct to pharmacological treatment, exercise can increase monoaminergic neurotransmitters and boost mobility (Carvalho 2018). While the majority of current research on exercise focuses on broad structural changes and neurotransmitter availability, our aim was to identify important molecular pathways linked to inflammation and mitochondrial function in the CNS.

Exercise and inflammation

Many inflammatory mediators are upregulated in chronic CNS disease states, including MS where levels of pro- and anti-inflammatory interleukins and tumor necrosis-factor alpha (TNFα) correlate with disease stage and severity (Nielsen, CH. et al 2016). Exercise has long been known to reduce inflammation in the periphery, including lowering the levels of the pro-inflammatory cytokines TNFα and IL-6 in metabolic disease states. (Pedersen, LR. et al. 2019). Similarly, in the CNS, high-intensity interval training decreases these cytokines in hippocampus of rats (Freitas et al. 2018) and treadmill running following cerebral ischemia reduces TNFα levels, (Janssens, K 2015, Nielsen CH 2016). Exercise also diminishes free radical damage in the

tissues of the brain (LaVoy et al, 2016). Another major mechanism for improvements is a reduction in chronic low-grade inflammation (Pedersen, L.R. et al 2019). However, research on the effects that exercise exerts on inflammation in the periphery has not given way to much of an understanding on the influence it might have on neuroinflammation and associated brain-metabolic changes. Chronic low-grade systemic inflammation is characterized as a 2- to 3-fold increase in pro-inflammatory markers that includes TNFα, ILs 1 and 6 and C-Reactive Protein (Pedersen, L.R. 2006). The proposed source of all of these factors in chronic low-grade systemic inflammation is not known, but it's agreed the source of TNFα is the adipose tissue. Because chronic low-grade inflammation arises from a variety of environmental factors and contributes to many common ailments including allergies, depression, diabetes, cardiovascular disease and arthritis, the pro-inflammatory milieu that characterizes it is relevant to our understanding of how exercise benefits the brain (Pahwa R, Jialal I. 2019).

In the CNS, the relationship between peripheral inflammation and central inflammation is just beginning to be examined. It was long thought that the blood brain barrier prevented immune factors in the periphery from entering the brain with the exception of certain diseases. However, we now know that common conditions such as sleep deprivation can increase blood brain barrier permeability and result in chronic low-grade inflammation centrally (Hurtado-Alvarado 2016). We sought to identify ways that exercise can alter the inflammatory milieu centrally. One such pathway that has been implicated through previous research in our laboratory is through decreases in reactive oxygen species and enhanced mitochondrial respiratory activity.

Inflammation, lipids and mitochondrial metabolism

Exercise can help peripherally by lowering the levels of the pro-inflammatory cytokines TNFα and IL-6 in metabolic disease states (Pedersen, L.R. 2019). Similarly, in the CNS, exercise decreases these cytokines in hippocampus of rats (Freitas et al. 2018). Because exercise can reduce blood-brain-barrier permeability and suppress activation of microglia through changes in neurotransmitter levels, it can reduce inflammation in the brain (Malkiewickz. 2019). Additionally, exercise has been shown to diminish free radical damage in the tissues of the brain (LaVoy et al, 2016) (Fig. 7). In addition, exercise is well known to influence the levels of circulating fats in the blood, but one lesser-known effect of physical activity is that it can alter the ratios of certain types of lipids within skeletal muscle. Lipid ratios are of great importance in CNS health because the brain has one of the greatest lipid concentrations of any organ (Walter & Fasbender. 2010). The class of lipids known as sphingolipids are one of the more prevalent found in brain tissue. Sphingolipids consist of the molecule sphingosine and fatty acid tails, and they may have various modified groups attached that determine their functions in membranes and within the cell (Christie, W.W. 2021). One of the more common types of sphingolipids found in the myelin membrane is sphingomyelin, which can be broken down into the subclass of lipid known as ceramides by the sphingomyelinase enzymes. Of particular relevance in neurodegeneration are the neutral membrane associated sphingomyelinase 2 (NSMASE2) and the acid sphingomyelinase (ASMASE), which are regulated by the TNFα1 receptor (TNFα1r) (Shamseddine, A.A. 2015) . More is known regarding NSMASE2 than ASMASE, but activation of either via TNFα can trigger a signaling cascade that initiates cell death (Walter & Fasbender. 2010) (Fig. 8).

Evidence that targeting the formation of ceramides from sphingomyelin degradation can lessen the severity of disease comes from an AD model where NSMASE2 deficient mice showed restored cognitive behavior in fear conditioning studies (Dinkins et al. 2016). Studies of MS have indicated that ROS release can activate the NSMASE2 and increase ceramide production, which is capable of activating phospholipase A2 and perpetuating inflammation (Walter & Fasbender. 2010).

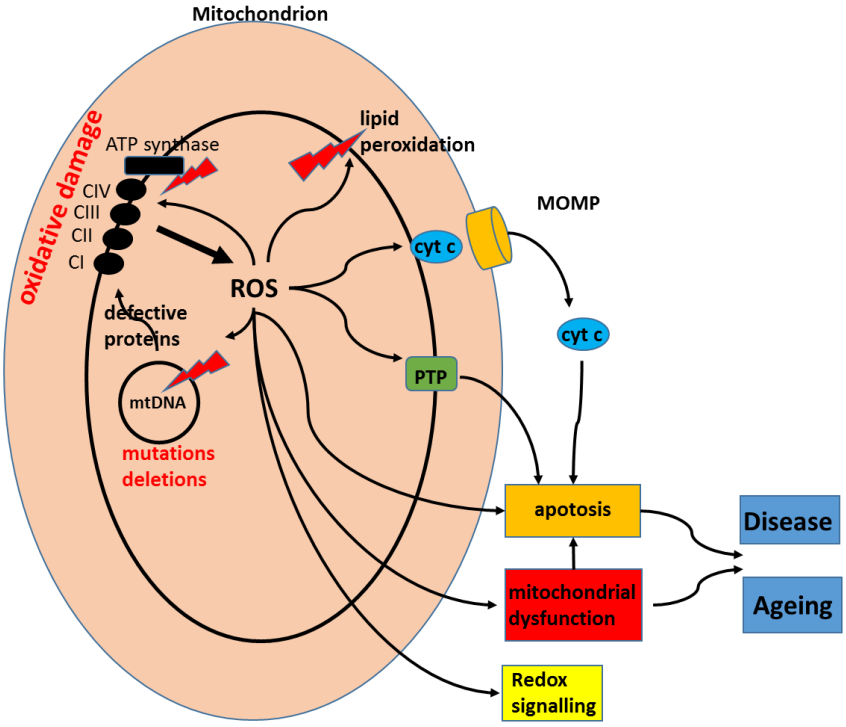


Figure 7. Mitochondrial associated ROS production. Cyclic pathway associated with ROS production and cell death and age-related diseases (M. Murphy 2009). Mitochondrial activity and environmental stressors lead to production of ROS, which then creates a cyclical pattern of inflammation and metabolic dysfunction within the cell.

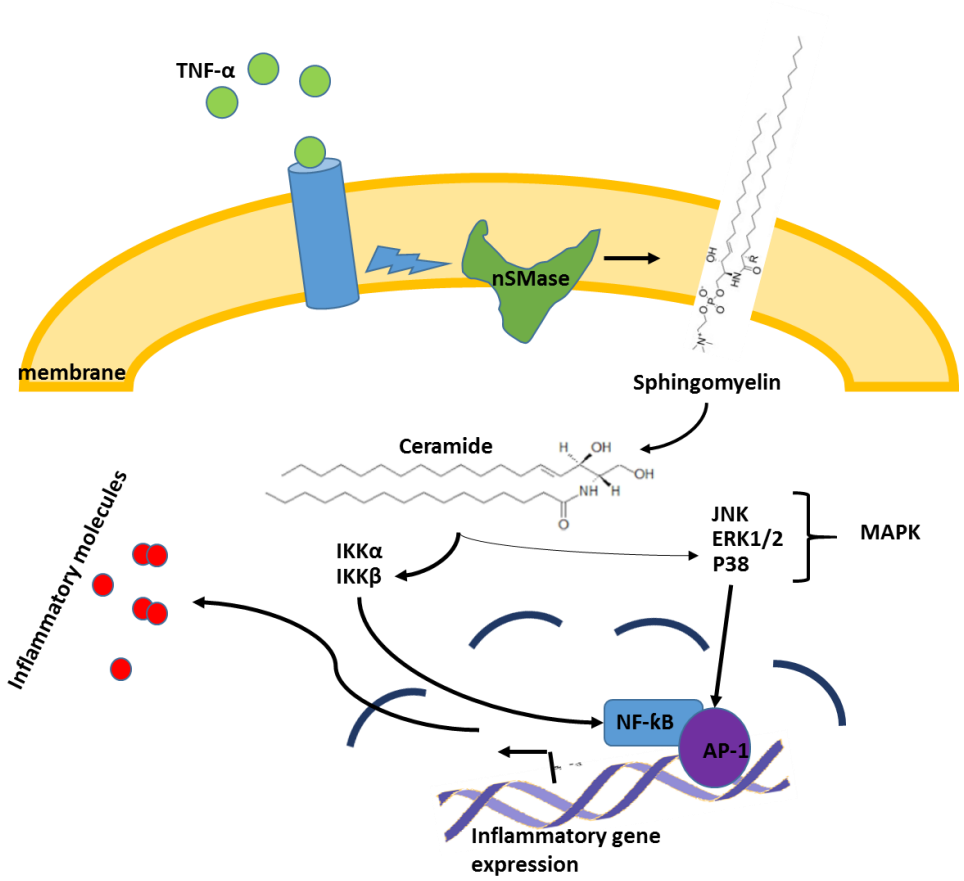


Figure 8. Cytokine -induced ceramide signaling. Ceramide pathway stimulation in response to binding of TNFα to the TNFαr1 receptor. (Al-Rashed et al 2020). TNFα binds its membrane receptor and stimulates activity of nSMASE, which breaks membrane sphingomyelin down into ceramides. These lipid species initiate NF-KB signaling and the release of inflammatory molecules that can lead to cell death.

Both moderate-intensity and interval training have been shown to decrease levels of the cytochrome C suppressor, Ceramide 18 (Sheperd SO, 2017) in the skeletal muscle of obese males, and treadmill running has been shown to suppress TNFα in joints in a rat arthritis model (Shimomura S, 2018). Physical activity is well known for its ability to reduce adiposity and improve blood glucose control. Thus far however, few studies have examined the effects of exercise on lipid species, aerobic respiration and inflammatory processes within the brain, especially not the long-term effects of voluntary physical activity. Because previous research in our lab has shown that both erythropoietin and hemoglobin beta repair mitochondrial dysfunction in disease and under hypoxic conditions, we wish to understand whether increases in these factors with exercise can influence the ceramide lipid ratios in the brain in a neuroprotective fashion.

Benefits of EPO in the brain

EPO is a well-known hematopoietic growth factor generally produced in the kidney, liver, and brain (Bunn et al 2013). However, while EPO is produced in the brain there is little evidence to suggest that exercise can enhance it expression centrally. Despite this, EPO and its receptor have been identified in neurons and glia. During brain development, EPO plays an important role in production and differentiation of neuronal precursor cells and causes differentiation of oligodendrocyte progenitor cells into mature oligodendrocytes. EPO also possess anti-oxidative, anti-inflammatory, and anti-apoptotic properties, and has demonstrated neuroprotective properties under hypoxic, oxidative, and ischemic conditions (Marti, H. 2004; Rey, F. et al 2019). These effects could be the result of increased Hbb expression, since endogenous EPO

expression or EPO injection induces neuronal Hbb expression and has been linked to increased neuronal survival under hypoxic conditions. Despite these links between EPO and Hbb expression there is currently little known regarding the ability of exercise to impact this mechanism. However, this approach provides a potentially effective strategy to improve brain health.

Potential relationship between neuronal hemoglobin and exercise

Exercise has long been known to reduce low-grade systemic inflammation and increase production of the oxygen carrier Hbb in the periphery, but less is known about the mechanistic benefits of exercise for the brain. Exercise can alleviate cancer fatigue through reductions in free radical damage and adjustments in the hypothalamic-pituitary-adrenal (HPA) axis (LaVoy et al, 2016), and similar benefits have been demonstrated for depression, anxiety and others. Cytokine increases, especially TNFα, can cause harmful production of reactive oxygen species (ROS) within the cell (Qiu X, et al 2019.) and inhibit mitochondrial metabolism. Additionally, increased ROS can activate neutral membrane sphingomyelinase 2, an enzyme that degrades specific membrane components to potentially detrimental fatty acid species (Dotson et al. 2015), which is of particular importance in the brain because of the high fat content in tissue there. It is well established that exercise increases levels of erythropoietin (EPO) in the periphery, and thereby increases Hb production (Ribeiro, 2017) which enhances oxygen-carrying capacity in the blood.

We have observed that the Hbb subunit is present in the nucleus of neurons and interacts with chromatin. The discovery of Hbb expression in neurons, until recently considered specific to red blood cells, is novel. While it has been established that hemoglobin is expressed in neurons in the rodent and human brain, the function of Hbb in these cells isn't clear. Previous studies support a role for Hbb in mitochondrial respiration in neurons (Richter et al., 2009; Schelshorn et al., 2009; Shephard et el., 2014). In a study by Biagoli et al., 2009 it was reported that dopaminergic cell lines over expressing Hbb exhibited changes in the expression of nuclear encoded mitochondrial oxidative phosphorylation genes. The focus of this study was to determine the impact of exercise on the expression of Hbb in the nucleus of neurons and to understand the role that Hbb plays in neuronal energetics and the oxidative environment within the CNS. In previous studies we have found that overexpressing the Hbb subunit in neuronal cell cultures can increase histone H3 trimethylation on lysine 4 (H3K4me3), a histone mark that activates transcription (Brown et al., 2016). Increasing H3K4me3 in neurons has been shown to activate transcription of mitochondrial genes and increase mitochondrial respiratory capacity (Singhal et al., 2015). Consistent with these findings, overexpressing Hbb with erythropoietin (EPO) in vivo, supports mitochondrial health and viability (Singhal et al., 2018). Chromatin fractionation studies in primary neurons show that Hbb is tightly complexed with chromatin and in situ fluorescence assays have revealed that Hbb interacts in the nucleus with the KDM5B histone demethylase that catalyzes the demethylation of H3K4me3 (Fig. 5). The KDM5B histone H3 demethylase is a 2-oxoglutarate dependent dioxygenase (2-OGDO) that requires O2 to oxidize C-H bonds (Vissers et al., 2014). Studies with a mutant Hbb construct containing a mutation that prevents O2 binding can be interpreted to suggest that Hbb

interferes with KDM5B by potentially sequestering O2 required for enzyme activity. This suggestion is further supported by our findings that the mutant Hbb still binds to chromatin and to KDM5B. Our data demonstrate that Hbb increases levels of H3K4me3 by inhibiting KDM5B mediated demethylation of H3K4me3. While we have demonstrated that Hbb is in the nucleus where it inhibits KDM5B and interacts with chromatin, there are still many questions regarding Hbb signaling in the nucleus that remain. In the proposed studies we will determine the impact of exercise on the Hbb expression and the ability to regulate histone methylation and gene expression. The effects of exercise on enhancing Hbb signaling as a potential approach for improving brain health and as a therapy for neurodegenerative disease will then be assessed.

Project Summary

Exercise has long been known to protect against cardiovascular diseases, as well as, reduce the risks of several cancers and metabolic disorders (Blair et al. 1999). In the central nervous system, exercise has also been linked to significant benefits related to neurodegenerative processes and has been linked to improvements in depression (Alkhadi. 2017) and cognitive function (Ahmed et al. 2017; Stranahan et al. 2012). In fact, exercise has been suggested to potentially be a disease-modifying approach to the treatment of Alzheimer's disease (Vreugdenhil et al. 2012; Law et al. 2018), as well as ameliorate the symptoms of both multiple sclerosis and Parkinson's disease (Doring et al. 2012; Frazzitta et al. 2013; Schenkman et al. 2018). However, the mechanisms responsible for the beneficial impact that exercise has on brain function are not well known. Our aim here is to identify important molecular pathways linked to the exercise-related benefits to CNS function. Many inflammatory mediators are

upregulated in chronic CNS disease states, including multiple sclerosis (MS) where levels of pro- and anti-inflammatory interleukins and tumor necrosis-factor alpha (TNFα) correlate with disease stage and severity (Nielsen, CH. et al 2016). Exercise has long been known to reduce inflammation in the periphery, including lowering the levels of the pro-inflammatory cytokines TNFα and interleukin-6 (IL-6) in metabolic disease states. (Pedersen, LR. et al. 2019). Similarly in the CNS, high-intensity interval training decreases these cytokines in hippocampus of rats (Freitas et al. 2018) and treadmill running following cerebral ischemia reduces TNFα levels, (Janssens, K 2015, Nielsen CH 2016). Exercise also diminishes free radical damage in the tissues of the brain (LaVoy et al, 2016).

Exercise has also been linked to increased levels of erythropoietin (EPO) and the consequent increase in hemoglobin (Hb) production (Ribeiro, 2017) in the periphery which enhances oxygen-carrying capacity of red blood cells. Again, in the CNS we have observed that EPO upregulates hemoglobin expression in neurons and restores Hb-beta (Hbb) levels in the cuprizone mouse model of MS (Singhal et al. 2018). While several studies have reported hemoglobin expression in neurons in the rodent and human brain (Biagoli et al., 2009; Richter et al., 2009; Schelshorn et al., 2009; Broadwater et al., 2011), the role of hemoglobin in neurons has remained elusive. We have identified a novel signaling pathway in neurons mediated by Hbb in the nucleus (Brown et al., 2016). Our data show that Hbb is tightly bound to chromatin and that Hbb signaling supports neuronal energetics. Further, we have linked expression of Hbb in neurons with increased levels of trimethylation of histone H3 on lysine 4 (H3K4me3), a histone H3 methyl mark that alters chromatin conformation and activates transcription (Brown et al., 2016). Our preliminary data show that Hbb expression activates transcription of nuclear

encoded mitochondrial genes and increases mitochondrial respiratory capacity. Therefore, we are interested in determining the impact of exercise on these potentially beneficial signaling mechanisms in the brain. Whether exercise can mimic the effects of EPO on Hbb expression and respiratory capacity of neurons remains to be determined. Further, exercise-induced enhancement in Hbb expression and impacts on mitochondrial respiration may also be linked to favorable changes in expression and action of inflammatory cytokines. Importantly, Hbb has intrinsic antioxidant properties and has been shown to scavenge hydrogen peroxide (H2O2), nitric oxide (NO), superoxide (OO-) and other harmful reactive oxygen species (ROS) that damage mitochondria (Gomes, I 2010). In addition to neuronal mechanisms, increased oxidative stress has been shown to increase the activity of neutral membrane sphingomyelinase 2 (NSMASE2), which degrades sphingomyelin contained with the myelin sheath to produce ceramides (Hannun & Obeid. 2008). Ceramides can have various effects based on their fatty-acid chain length, many of them harmful. Ceramide lipid ratios are altered in a number of neurodegenerative conditions including MS and Alzheimer's (Fyrst, H 2010). Therefore, exercise may improve mitochondrial respiratory capacity in neurons, induce favorable alterations in the pro-inflammatory environment and reverse detrimental changes in ceramide lipid ratios.

Aim 1: Voluntary wheel running increases hemoglobin beta in neurons

We have hypothesized that exercise will increase hemoglobin beta in neurons. Increases in mitochondrial and nuclear hemoglobin beta will occur with exercise and will be associated with increased H3K4Me3 in neurons

Aim 2: Increased hemoglobin beta is correlated to improved mitochondrial respiration and decreased inflammation in brain cells

In this aim we will test the hypothesis that exercise will improve mitochondrial metabolism. We expect increased expression of several nuclear-encoded mitochondrial respiratory factors and improved mitochondrial respiratory capacity in neurons.

Aim 3: Exercise increases Hbb and EPOR in neurons and regulates expression of mitochondrial genes

We have hypothesized that changes in mitochondrial metabolism will occur in conjunction with changes associated with activity of the EPO pathway, such as upregulation of EPOR, increased H3K4me3 levels and altered expression of candidate genes previously identified using chipseq for H3k4me3.

This study focused on examining the connection between exercise and brain hemoglobin beta production. We looked at expression levels of Hbb in different cell fractions taken from the cortex of rat brains after five and seven weeks of voluntary wheel running. We considered the volume of running that the animals performed on average and attempted to correlate that to Hbb expression and benefits to the cell such as mitochondrial respiration. We found some increase in mitochondrial respiratory capacity in running animals as well as significant changes in Hbb expression in nuclear cell fractions when compared to sedentary controls. We attempted to delineate how these changes occur by examining the expression of mitochondrial respiratory complexes, the activator of mitochondrial biogenesis, PGC1a, fission protein DRP1

and levels of the histone methylation marker, H3k4me3. Additionally, we looked at inflammatory factors that might be linked to these mitochondrial changes including TNFa and ceramides. Because previous research in our laboratory has shown that the cytokine erythropoietin increases Hbb in the brain and can increase respiratory complexes in the cuprizone mouse model of MS, we looked at expression of erythropoietin receptor in rat cortex. Initial examination of ceramides by thin layer chromatography were inconclusive, but hinted at a trend for running animals to have lower cell ceramide content, similar to what is seen in skeletal muscle tissue with exercise. No consistent changes in mitochondrial complexes were seen with western blot, but PGC1a and DRP1 were both changed in the running group. Nuclear Hbb increases were seen in both mid- and anterior- cortical slices, and these results were replicated with western blot from cortical tissue across many trials. EPO receptor was increased in running animals, suggesting that some of the metabolic changes seen in the running group occur through upregulation of this receptor in the brain. Consistent with studies on EPO administration in the brain, we saw an increase in the neuronal mitochondrial activity marker NAA in our running group. However, H3k4me3 results were inconclusive, suggesting that methylation patterns played a larger role in the changes we saw than the levels.

Chapter 2

Voluntary exercise increases hemoglobin beta in rat cortex

Background

Conditioned athletes have long been known to have increased hematocrit levels and enhanced oxygen-carrying capacity supporting improved physical performance. In addition, exercise has long been known to protect against many different diseases, including cardiovascular disease and all-cause mortality (Kodama et al. 2009), as well as, hypertension, stroke and metabolic disorders (Blair et al. 1999). Many studies lately have also begun to provide exciting evidence demonstrating link between exercise and significant benefits related to neurodegenerative processes (Alkhadi et al. 2017; Ahmed et al. 2017; Stranahan et al. 2012). It has been suggested that this benefit occurs in part due to increases in the production of neurotrophic factors, including BDNF (Cotman and Engesser-Cesar 2002; Cotman and Berchtold 2002), reductions in oxidative stress (Radak, Z. et al. 2016) and neuroinflammation (Kohman et al. 2013; Barriento et al. 2011). Further, more recent studies have identified pathways that involve EPO signaling as an important mechanism that supports improved cognition and neurogenesis (Wakhloo et al. 2020). Our study sought to examine if exercise can increase hemoglobin production in nonerythroid cell types in the brain, in particular neurons, since several groups have previously found hemoglobin to be produced endogenously throughout various brain regions (Brown, N. et al 2016; Biagoli M. et al. 2009). Previous data from our lab has shown that hemoglobin coprecipitates with multiple mitochondrial proteins and redox sensing proteins that act as transcription factors (Broadwater et al. 2011) In other studies, treatment of mice with EPO

resulted in increased Hbb and increased H3K4me3 (Singhal et al. 2018). Along with these changes an increase in expression of mitochondrial complex proteins and levels of NAA, an important marker for neuronal mitochondrial activity amongst other important functions, were noted. We suspect that Hbb can affect transcriptional activation through altering H3K4me3 patterns and by changing expression of mitochondrial genes. In these studies, we observed that H3K4me3 levels were increased two-fold in Hbb transfected SH5Y cells. It is still unclear what the primary function of endogenously produced Hbb is, but it is likely that it serves as more than an oxygen transport molecule given its ability to interact with the redox sensing demethylase KDM8 and histone H3, a combination that implies Hbb mediates transcriptional regulation, either directly or through other proteins (Brown et al, 2016). Thus, Hbb may serve to link epigenetic signaling pathways with cellular energetics and could potentially play a role in neuroprotection.

Thus, in these studies we examined the impact of physical activity on the expression of Hbb in the brain of rats. Adult rats were provided unrestricted access to running wheels for seven weeks based on data from studies on stress showing that time points earlier than six weeks were insufficient for exercise to exert its benefit (Greenwood, B.N., et al. 2005). After, we examined the expression of endogenous Hbb in neurons of the cortex.

Materials and Methods

Animals

Age and gender-matched groups of Sprague Dawley rats (5 running; 5 sedentary) between the ages of 8-10 weeks were provided access to a running wheel for seven weeks. Seven weeks

was selected since previous studies demonstrated significant increases in hippocampal neurogenesis within this time frame and it was suggested that the most effective response was obtained if exercise was aerobic and sustained (Nokia et al. 2016). In one group the wheel was permitted to rotate freely and animal's activity was monitored by documenting number of rotations each morning after the evening bout of activity (running group). The other group were also provided with a wheel in their cages but the wheel was fixed and unable to rotate. Animal weights were recorded at the start and end of study. Running activity was recorded using odometers attached to the wheels and average daily and weekly running volume was determined. Cages were surrounded by opaque plastic bins so rats could not view each other, and former cage mates were placed in separate groups. All animals were housed and fed according to Kent State University's IACUC protocols.

At the end of the running period, animals were anesthetized using a chamber filled with isoflurane. The animals were pinned to a dissection board with a small beaker filled with isoflurane-soaked cotton over the head to maintain anesthesia. The abdomen was cut down the center and the diaphragm moved aside to gently expose the apex of the heart. A 21g needle was inserted at the apex in the direction of the left ventricle, and a peristaltic pump filled with 0.9% saline solution was turned on at 0.3-0.5 flow rate. The hepatic portal vein was severed upon beginning to allow blood to drain. When abdominal organs and eyes appeared white, the flow was stopped and brains were removed over ice. Brains were divided in half and placed in tubes on dry ice for protein, NAA and lipid analysis, while the other half was placed in a tube containing 4% paraformaldehyde and stored on ice. Frozen brain cortices were dissected

and split for use in a subcellular fractionation method for nuclear, cytoplasmic and mitochondrial proteins or Bligh Dyer method for lipid extraction.

Western Blot

Protein was isolated from the cortex of one hemisphere (100-150 mg) was taken for protein fractionation as previously described (Dimauro et al. 2012). Tissue was homogenized with a mini-homogenizer for 60s in five volumes of extraction buffer. Protein content of each fraction was determined using the Bradford Assay. Proteins in each fraction (30-50 ug) were separated using SDS polyacrylamide gel electrophoresis on NuPage 12% Bis-Tris gels (Invitrogen) before transfer to nitrocellulose membranes. Membranes were blocked for 45 minutes at 23C in 5% BSA/TBST before overnight incubation in primary antibody at 4C on a 3D rotator. The membrane was washed one time for 10 minutes at 23C in TBST pH 7.9 and then incubated at 23C for 45 minutes in the appropriate HRP-conjugated secondary antibody. Immunoreactivity was detected with Luminol (Santa Cruz Biotechnologies). Fraction purity was determined by Western blotting with antibodies to cytosolic (GAPDH), mitochondrial (ARALAR) and nuclear (NeuN) markers. Western blots were run with the monoclonal antibody to Hbb (Abcam) and relative levels were determined after normalization to GAPDH, or ARALAR or H3 for cytoplasmic, mitochondrial or nuclear fractions, respectively. Protein levels were determined from at least three separate experiments by densitometry using ImageJ. A Student's T-test was used to determine statistical significance of changes in protein levels using $P \leq 0.05$ as significant.

Fluorescent imaging

Half brains previously fixed overnight in 4% PFA and stored in 1X PBS at 4C were cryoprotected in a 10-60% sucrose gradient and sliced with cryostat at 100 microns. Sections taken from anterior and mid-frontal brain regions were boiled for twenty minutes in sodium citrate solution and blocked for 45 min in 3% Normal Donkey Serum containing 1% Triton-X 100.

To visualize Hbb immunoreactivity, tissue sections were incubated in mouse anti-Hbb (1:100; monoclonal) (Sigma) and chicken anti-NeuN (1:500) (Millipore Bioscience Research Reagents). All incubations were done in PBS, 1.0% Triton X-100, and 3% donkey serum overnight at 23°C. Tissue sections were then incubated in donkey anti-mouse Alexa-488 (1:1000) and donkey anti-chicken Alexa-555 (1:1000) for 2 h at 23°C. Both secondary antibodies were purchased from Invitrogen. Sections were washed and mounted with Vectashield mounting medium containing DAPI to label nuclei. Images were acquired with an Olympus Fv1000 confocal microscope equipped with five laser lines (HeCd 442 nm, Ar 488 and 514 nm, HeNe 543 nm and HeNe 633 nm). Images were viewed with ImageJ (National Institutes of Health) and channels merged to show colocalized signals. Relative fluorescence was compared in running and sedentary tissue blocks. Images were captured sequentially for each channel to prevent bleed through and spanned the sections. An image mask was created using the NeuN channel as a guide to include the entire nucleus. The thresholded image mask was then used to clip the Hbb channel, and the pixels within the unclipped region were summed. This technique measures the amount of Hbb fluorescence from within individual NeuN-stained nuclei. Mean density of Hbb immunofluorescence was obtained from the average intensity from 6-12 NeuN-positive neuronal nuclei from at least 3 different images per sample.

Statistics

Data are from at least 4-6 rats per group (running or sedentary). Excel was used to calculate averages, standard deviations, standard error and variance. Variance was used to determine whether a homoscedastic or heteroscedastic t-test was needed. One-tailed two-sample t-tests, selected according to expectations we had for the direction of the change, were used to determine the difference between means.

Results

Running volume

Rats were provided unrestricted access to running wheels for 7 weeks. The number of revolutions were recorded and documented daily and distance traveled was calculated for each animal. Fig 9 illustrates the average daily distance traveled for individual rats which ranged from approximately 0.25 km to 6.2 km per day. Most animals covered approximately 2-3 km/day with an average for all animals of 2.94 km/day.

Hemoglobin expression in the cortex in response to running

We have previously demonstrated the expression and distribution of hemoglobin in the rat brain and the morphological analysis indicates that the majority of the expression is localized to neurons (Li et al. 2015). Further, particularly intense staining was observed in the cortex and hippocampus as well as other regions (Li et al. 2015). It has also been established that exercise increases levels of erythropoietin (EPO) in the periphery and is linked to increases in

hemoglobin production. (Riberio et al. 2017) and while there is a link between physical activity and hemoglobin expression in the periphery it is not known if similar changes can be observed in the brain. Thus, we examined whether levels of Hbb were changed in response to running. To determine the changes in the subcellular expression of Hbb, tissue samples separated into cytosolic, mitochondrial and nuclear fractions from cortical gray matter isolated from running or sedentary rats. Western blotting was performed on these fractions. The purity of the fractions was determined using antibodies to the cytosolic protein GAPDH, the mitochondrial membrane protein ARALAR and the nuclear marker H3.

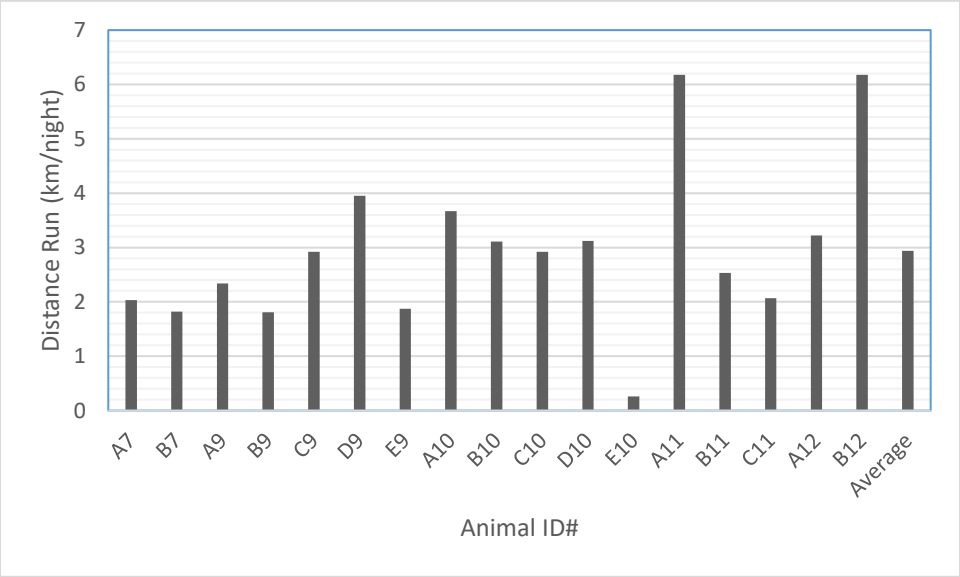
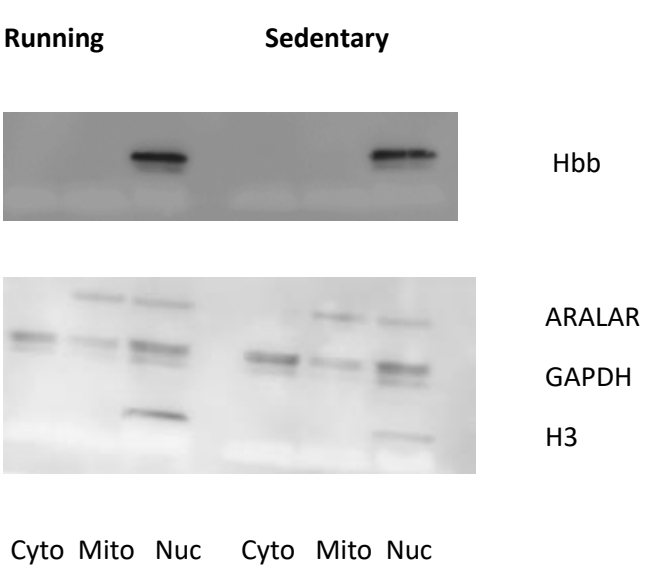


Figure 9. Running volume. Distance run by individual rats each day. Rats were provided unrestricted access to running wheels (33 cm circumference) for 7 weeks and revolutions recorded using an odometer. Average distances run over the 7-week period (kilometers: km) are reported for individual animals with an average of all animals reported as 2.94 km/day.

A representative blot illustrating the purity of the fractionation is shown in Figure 10A. There is some contamination between the mitochondrial and nuclear fractions. However, only the nuclear fraction contains the nuclear marker (H3). Further, as can be seen there was no significant detectable Hbb in either cytoplasmic or mitochondrial fractions obtained from tissue samples of rat cortex. In contrast, there was strong staining for Hbb in nuclear fractions which was increased by approximately 30% in fractions isolated from the cortex of running animals when compared to sedentary controls (Figure 10B).

A.


Running
Sedentary
Hbb
ARALAR
GAPDH
H3
Cyto Mito Nuc Cyto Mito Nuc

B.

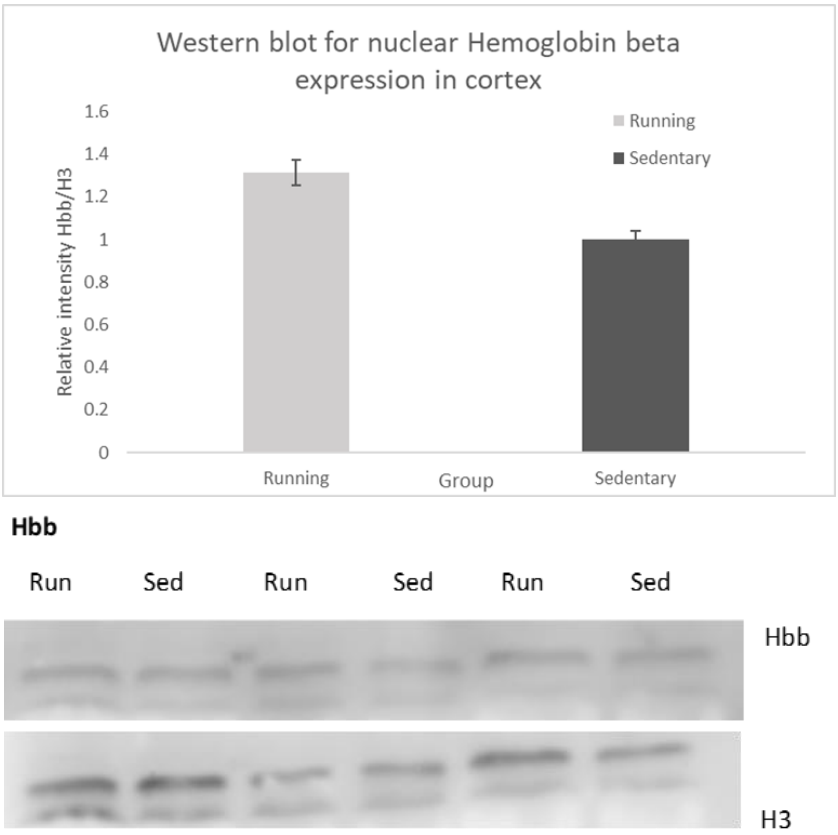

Western blot for nuclear Hemoglobin beta expression in cortex
Relative intensity Hbb/H3
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Running
Sedentary
Running
Group
Sedentary
Hbb
Run Sed Run Sed Run Sed
Hbb
H3

Figure 10. Hemoglobin expression in rat cortex. Hbb subcellular localization is shown in part A. Top: Representative blot shows that Hbb is present mainly in the nuclear fraction of cortical tissue. Bottom: Cytoplasmic (Cyto), mitochondrial (Mito) and nuclear fractions (Nuc) blotted for purity. GAPDH was the only marker identified in Cyto fractions. In Mito fractions ARALAR was identified as was GAPDH. In Nuc fractions H3 was identified along with both GAPDH and ARALAR. The presence of additional bands in both Mito and Nuc fractions suggests some contamination in the fractionation.

In (B), Nuclear hemoglobin expression is shown. Top: Quantitation shows that average Hbb expression in nuclei of running animals tended to be increased compared to sedentary animals however, this did not reach statistical significance. (N=9 for each group, p=0.06). Statistical analysis was determined by Student T-test. Error bars represent SEM. Significance determined at $P \leq 0.05$. Bottom: Representative western blot of Hbb expression in cortical samples showing increases expression of Hbb in nuclei from running animals compared to sedentary animals.

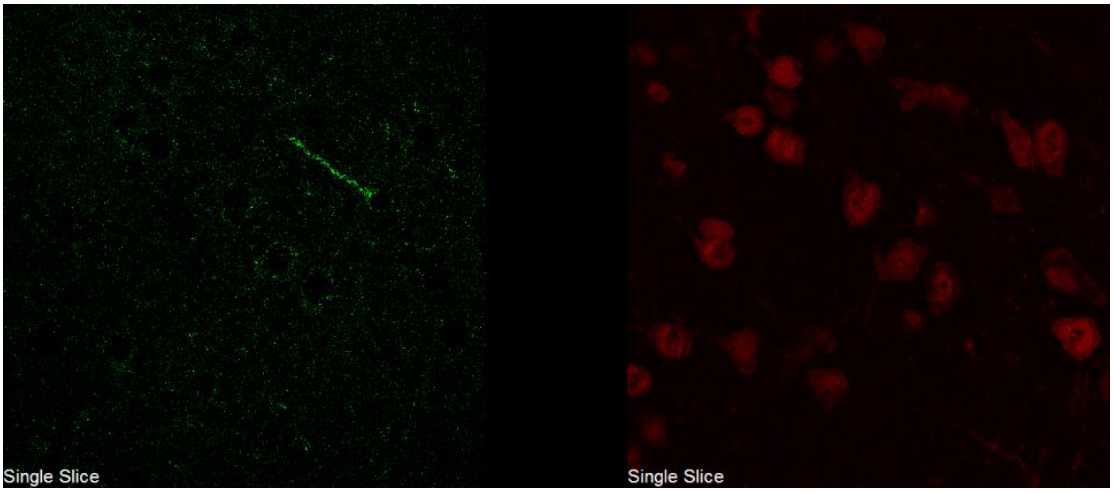
We then determined changes in hemoglobin beta expression in the nuclei of neurons from brain slices taken from multiple cortical regions (Figure 11 and 12). To more specifically localize the presence of Hbb in neurons, we performed immunohistochemical staining of cortical slices from multiple brain regions (mid and anterior frontal cortex) with specific monoclonal antibodies to Hbb and the neuronal nuclear marker NeuN. The stain for hemoglobin beta (green) had high background, as is typical for blood proteins, but nuclei were clearly outlined with stain. This was found to be consistent throughout the majority of the outer cortex in both groups (Figures 11 and 12). Hbb was predominantly observed in the and around these neuronal nuclei and staining intensity was increased in slices obtained from running animals compared to sedentary controls. This is consistent with staining we have observed both in brain and in neuron cultures (Singhal et al. 2018). Interestingly, this may suggest that Hbb is capable of forming stable homotetramers in the nucleus and this form has actually been reported to possess a higher binding affinity for O_2 than the $\alpha 2\beta 2$ heterotetramer (Bellelli et al. 2006). The fluorescence of hemoglobin (green) was compared to the expression of the neuron marker NeuN (red) and then data was corrected for area. Images were selected for clarity, and between six and twelve images were used to quantitate fluorescent intensity for each sample. For the mid-cortical data, twelve data points were used per sample due to the faintness of the staining and the potential for ambiguity to arise. Six data points were used for the anterior region, which stained more clearly, however the data was collected using the exact same method. Both sets of data showed a marked increase in hemoglobin beta localized to nuclei in running animals compared to sedentary controls. Fluorescent intensity for nuclear Hbb staining

was increased in these cortical regions by approximately 45% to 80% in slices isolated from running animals when compared to sedentary controls (Figures 12C and 12D respectively).

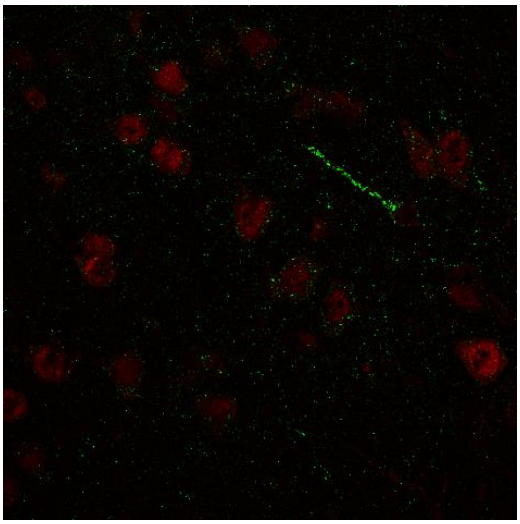
Running

A.

Hbb **NeuN**



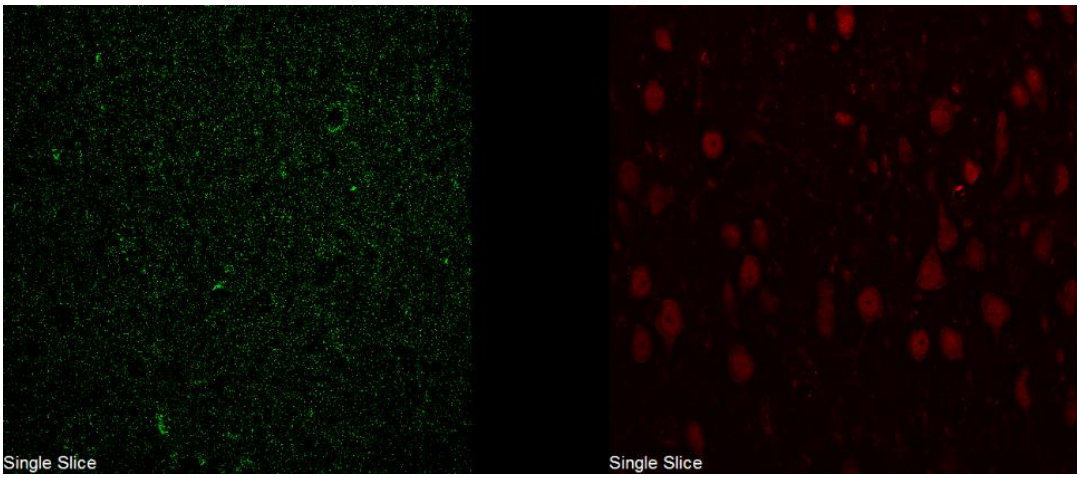
Combined



Sedentary

B.

Hbb **NeuN**



Combined

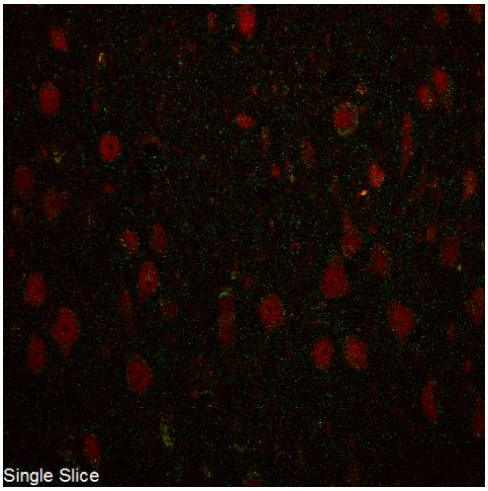


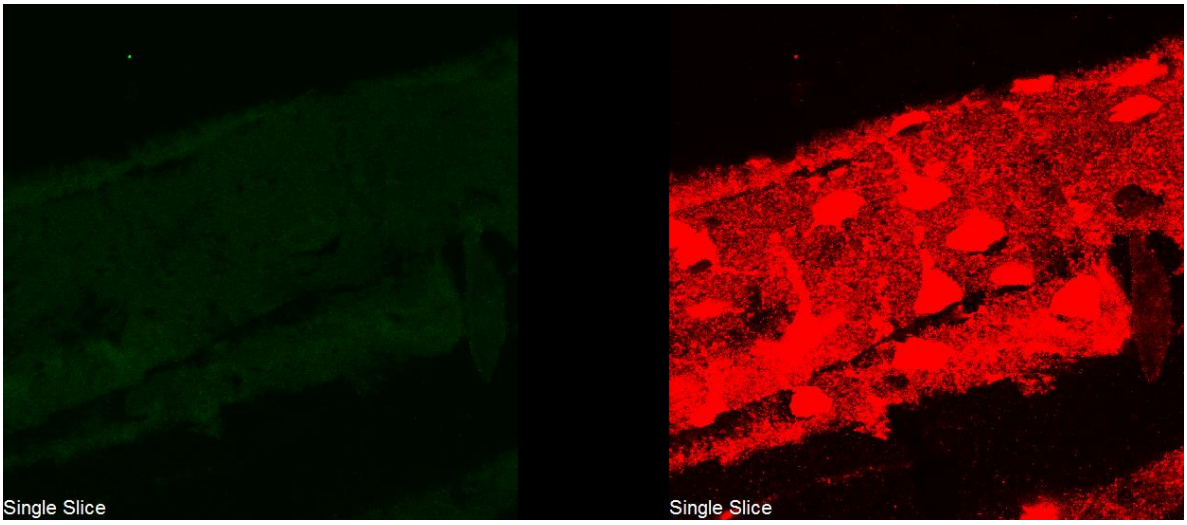
Figure 11. Changes in Hbb expression in mid frontal cortex in response to running. Slices are from mid-frontal cortical brain regions (100um sections, post-antigen retrieval) from a running animal (A top) and sedentary animal (B bottom). Sections were stained with Hbb (1:100: green) and NeuN (1:500: red). The slides were imaged with confocal at 60X magnification. Fluorescent intensity was measured with ImageJ and the area of the stain was accounted for by dividing the mean density by it before finding the ratio of Hbb to NeuN. The mean fluorescent intensity was

0.84 ± 0.11 in the running group and 0.55 ± 0.11 in the sedentary group (n=7 per group).

Student t-test $P \leq 0.05$.

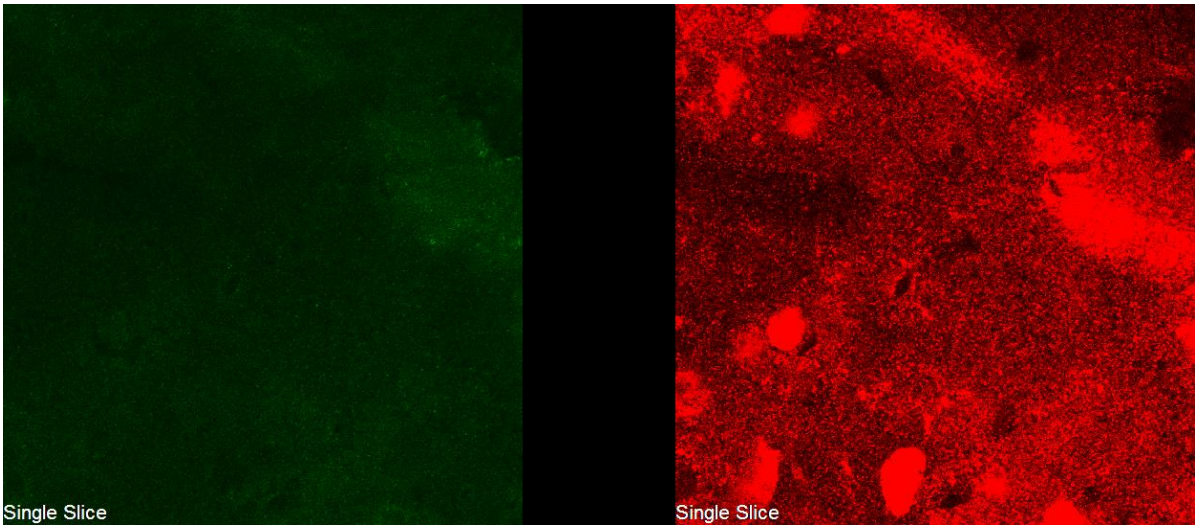
Running

A

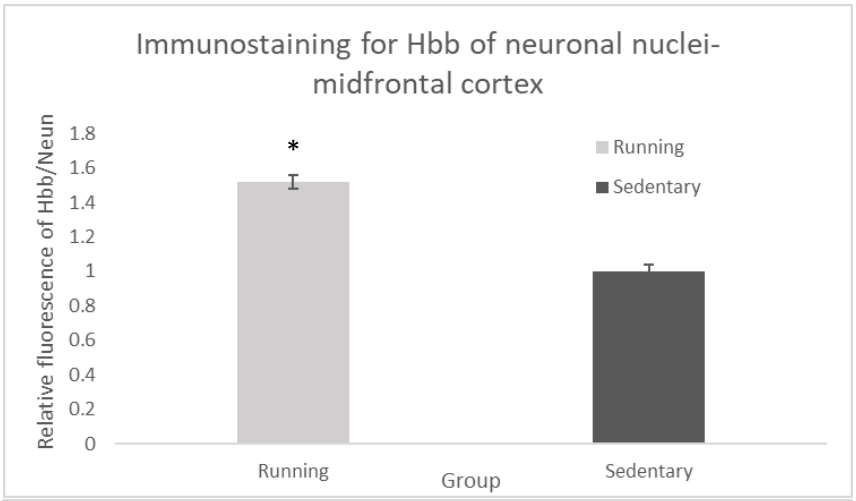


Sedentary

B



C



D

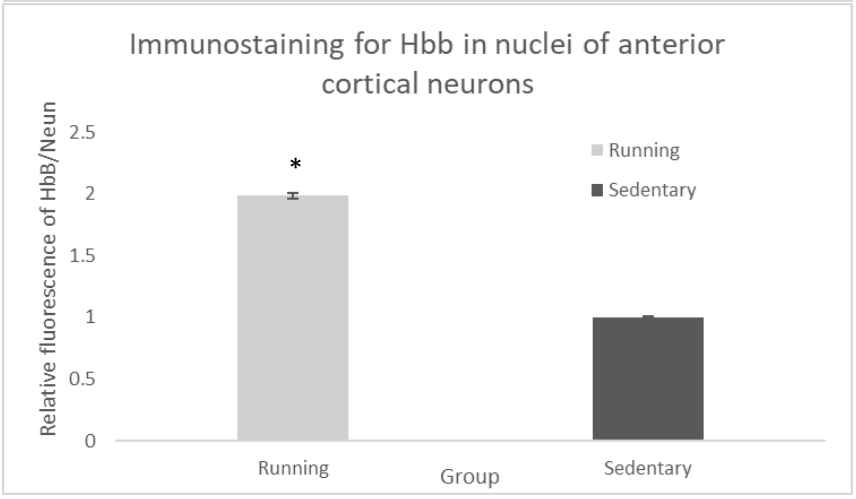


Figure 12. Changes in Hbb expression in anterior cortical neurons in response to running. Slices are from anterior-frontal cortical brain regions (100um sections, post-antigen retrieval) from a running animal (A top) and sedentary animal (B bottom). Sections were stained with Hbb (1:100: green) and NeuN (1:500: red). The slides were imaged with confocal at 60X magnification. Fluorescent intensity was measured with ImageJ and the area of the stain was accounted for by dividing the mean density by it before finding the ratio of Hbb to NeuN.

Quantitation is shown in C, D. The mean fluorescent intensity was 0.29 ± 0.06 in the running group and 0.14 ± 0.014 in the sedentary group (n=8 per group). Student t-test * $P \leq 0.05$.

Figures 12C, D. The relative expression of Hbb was determined by fluorescent confocal microscopy. Fluorescent intensity was determined in slices obtained (n=7) and six to twelve images were used to quantitate intensity, as described in methods. Mean fluorescence was 0.84 ± 0.11 in the running group and 0.55 ± 0.11 in the sedentary group, $P<0.05$. C) Represents average fluorescent intensity in mid-frontal cortex. D) Represents average fluorescent intensity in anterior-frontal cortex (0.29 ± 0.06 in the running group and 0.14 ± 0.014 in the sedentary group, * $P<0.05$).

Conclusions

Animals in our running groups tended to run an average of 2.5KM per night, and runners had lower weights on average than the sedentary animals as would be expected (mean weight at the end of the study was 335.2g vs 352.6g, n=10, respectively), but the difference was not significant. There were no unexpected deaths, injuries or illnesses in either group in any of our trials. Our seven week running trials showed a strong trend for Hbb to increase in the nuclear fraction of cortical tissue by Western blot (Figure 10). With immunohistochemistry, we were able to confirm a significant increase in Hbb in the nuclei of cortical neurons. It is likely that Hbb may also be present in other cell types within the cortex, accounting for the different results with these methods. Future studies will have to look more closely at the distribution of Hbb in non-neuronal cells to fully explain the inconclusive western blot results.

Chapter 3

Increased hemoglobin beta is correlated to improved mitochondrial respiration and decreased inflammation in the brain

Background

Previous research in our lab has shown a strong relationship between hemoglobin expression and mitochondrial function in neurons (Dutta et al. 2006; Pandit et al. 2009; Campbell et al. 2011; Broadwater et al. 2011; Witte et al. 2013). This is of relevance to neurodegeneration and inflammatory conditions that affect mitochondrial respiration such as occurs in AD, PD and MS (Norat, P. et al. 2020). Neurons and glia have high energy demands and are particularly susceptible to alterations in mitochondrial energetics, since this can lead to disruptions in ion homeostasis, production of ROS and increased inflammatory activity (Kann & Kovacs, 2007). Consistent with this, several studies have used magnetic resonance imaging (MRI) and spectroscopy (MRS) to document that the marker for neuronal mitochondrial activity, NAA, is decreased in diseased brain. Further this decrease has been shown to proceed the subsequent reductions in brain volume suggesting that disruptions in mitochondrial respiration likely proceeds neurodegeneration (Ge et al. 2004; Cader et al. 2007). Reductions in mitochondrial respiration have been shown to contribute to neuronal and axonal pathology in neurodegenerative disease by impacting electrical conduction and axonal transport mechanisms (Waxman 2006). Mitochondrial health is complex, and injury to mitochondria triggers pathways within the cell related to redox state. When inflammation is present, ROS production in mitochondria can be triggered through the TNFα1 receptor. Activation of this receptor initiates degradation of membrane sphingomyelin to ceramides, nitrous oxide (NO)

production and caspase cascades. This leads to increased ROS signaling within mitochondria and decreased respiratory capacity. In addition, increased ceramide production can damage the mitochondrial membrane and trigger cytochrome c release and apoptosis. However, the relationship between mitochondrial health and inflammation is reciprocal. Tipping the odds in favor of efficient respiration by increasing antioxidant levels or expression of mitochondrial complexes can lower ROS signaling and preserve the health of cells. Previous work in our lab has shown that EPO can raise levels of the neuronal respiratory marker NAA, increase the expression of mitochondrial complex III protein subunits and increase respiratory capacity in the brains of cuprizone treated mice (Singhal et al. 2018). Whether or not exercise can alter similar pathways as described above is not known. However, we have seen that exercise increases Hbb expression in neurons, and others have demonstrated that exercise increases EPO expression in the periphery and the brain. Therefore, we examined the effect of exercise on measures of mitochondrial function, including respiration, mitochondrial metabolism and the impact on inflammatory markers. Thus, we looked at mitochondrial respiration and NAA production in the tissues of running animals vs the sedentary controls and chose to look at the expression of the nuclear encoded mitochondrial respiratory complex protein Cox5b. This subunit is a part of cytochrome c oxidase, the complex where O_2 is reduced to H_20 in the final step of electron transport, and believed to be to be the source of most ROS. In addition, we also examined changes in markers of mitochondrial health using DRP1, a mitochondrial fission protein and PGC-1α, a marker of mitochondrial biogenesis. Lastly, we looked at ceramide lipid content, and TNFα cytokine in brain tissues from these cohorts.

Materials and Methods

Respirometry

Fresh un-perfused half brains were processed immediately for the mitochondrial stress test to compare respiratory capacity in running vs. sedentary animals. Mitochondria were isolated using a density dependent centrifugation method, according to the Agilent mito stress test protocol. The night prior to the study, a calibration plate was warmed at 37°C and the Seahorse machine was turned on. The morning of, the standards were prepared and the assay was calibrated. 150mg of cortical tissue was homogenized with a dounce homogenizer in 500ul of MSHE+BSA buffer. Homogenized tissue was put through a series of centrifugations to isolate mitochondria, and a Bradford assay plate was prepared during the wait time.

The final pellet was resuspended in minimal volume of MSHE (~50ul) and 5ul was taken from each for Bradford assay. Protein concentration was estimated at 480nm and the dilution needed for 5ug of mitochondria per well was determined using the standard curve. 5ul of 1ug/ul stock + 45ul of 1X MAS + Substrate was added in each well, in duplicate or triplicate for each sample, while the plate was on ice. Then 450ul of warmed 1X MAS + Substrate was added to each well. Finally, the plate was spun down at 2000g for 20 minutes at 4°C. Oxygen capacity analysis was performed using the template in the Wave Seahorse Software. Data was analyzed in excel using a Student's t-test to look at the difference between the mean rate of oxygen consumption from each group.

Protein fractionation and Western Blot

Western blots using mitochondrial protein fractions from rat running trials were probed for COX5b, PGC1α and DRP1 using 50ug of protein and normalized to ARALAR for each respective fraction as described previously for the other experiments. Samples from 4-8 animals per group were used, and ImageJ was used to quantify differences in expression between the two groups. Data were analyzed with a Student's t-test with $p< 0.05$ considered significant.

HPLC for NAA

The neuronal mitochondrial metabolite NAA was quantitated in cortical tissue from rats using HPLC (n=at least 6 per group). For brain tissue, NAA was quantitated from gray matter from both brains obtained from running and sedentary rats. For HPLC, 100mg of brain tissue was homogenized in ice-cold 90% methanol using a dounce homogenizer. The samples were centrifuged twice at 14,000 RPM for 10 minutes at 4°C, and supernatant was transferred to a new tube each time. The final supernatant was dried with a speed vacuum and powder was dissolved in 0.5mL of deionized water before adding this solution to pre-washed polyprep pre-filled chromatography columns (Bio-Rad, Hercules, CA). The column was washed with 1ml of deionized water and the eluant was collected and lyophilized overnight. Each sample was reconstituted in 400ul of deionized water. HPLC was performed using a Whatman partisil 10 SAX anion-exchange column (4.6 mm x 250 mm) in an Agilent 1100 Series HPLC Value System (Agilent Technologies, Santa Clara, CA). Two liters of a mobile phase consisting of 0.1 KH_2PO_4 and 0.025M KCL pHed to 4.5 in HPLC grade water wash prepared and added to the column after

washing it with 20-30 volumes of 10% methanol in deionized water. Samples were mixed and 200ul of each sample was loaded in duplicate into HPLC vials. Retention data was collected at a flow rate of 1.5mL/min and monitored with Agilent 1100 at 214nm. Retention time was originally determined to be 5.10min using an NAA standard. Peak areas were acquired with Agilent Chemstation software. NAA levels in nmol/mg in triplicate and average level was compared between groups using a Student's t-test.

Lipid isolation and Thin Layer Chromatography

Thin layer chromatography was performed for sphingomyelin and ceramides. A modified Bligh-Dyer protocol was used to extract lipids from the rear cortex of rat brain (approximately 100mg of tissue). First tissue samples were homogenized in 1mL of cold 1X PBS over ice in a dounce homogenizer. A solvent system of chloroform/methanol/water and centrifugation was used to separate out the lipids. The chloroform layer containing only lipid was removed using a Pasteur pipette with bulb, dried under nitrogen and stored in glass vials at -20°C, tightly capped and sealed. To classify and relatively quantify ceramides and sphingomyelin, lipids dissolved in chloroform were loaded onto glass silica plates (~30ul per sample, or as determined for each class of lipid) and dried briefly under nitrogen. A chamber equilibrated for at least 30 minutes with a 65:25:4 Chloroform: Methanol: Water solvent was used to run the lipids in order of hydrophobicity. One-dimensional separation on plates was analyzed using standards for the lipid classes of interest. Once complete, the plates were stained with either iodine or sulfuric acid to visualize lipid spots.

High performance liquid chromatography/mass-spectrometry was performed on later samples by Dr. Shriver at Akron University to look at specific chain lengths of ceramide present and allow analysis of ratios between the two groups.

ELISA for TNFα

Cytoplasmic protein fractions prepared using our standard protocol were used to obtain 1-2ug of protein for rat TNFα ELISA assay. The wells of the uncoated plates were filled with 100ul of capture antibody in coating buffer (purified rat TNFα antibody at 1:250 concentration in 1X PBS) and covered for an overnight incubation at 4°C. The capture antibody was removed and wells were washed 3X for at least a minute with wash buffer (0.05% Tween in 1X PBS) and then blocked for one hour at 23°C with 1X ELISPOT diluent. The wells were washed at least once with wash buffer and the standard curve for rat TNFα was prepared using serial dilution method. The samples were added in 100ul volume and the plate was sealed and incubated overnight at 4°C. Wells were washed again 3-5 times with wash buffer and the detection antibody in ELISPOT diluent was added. The place was incubated for an hour at room temperature and then washed 3-5 times with wash buffer before the addition of the avidin-HRP enzyme. After a 30-minute incubation at 23°C and then washed 5-7 times in wash buffer. Finally, TMB solution was added for 15 minutes and the reaction was stopped with 1M phosphoric acid. Optical density was read with the spectrophotometer at 450nm and 570nm. 570nm values were subtracted from 450nm values in order to be used in equations that determine the concentration using the standard curve.

Statistics

Excel was used to calculate averages, standard deviations, standard error and variance. Variance was used to determine whether a homoscedastic or heteroscedastic t-test was needed. One-tailed or two-sample t-tests were selected according to expectations we had for the direction of the change, were used to determine the difference between means.

Results

NAA concentration in running animals

We examined the effects of running on mitochondrial health by quantifying levels of NAA, the marker of mitochondrial activity, using a methanol:water solvent dependent form of HPLC. NAA is essential to signaling between neurons and oligodendrocytes to affect myelination. In general, NAA levels can be a good indicator of metabolic state within the cell, with higher levels correlating to better health. The HPLC software was calibrated to measure the time that the peak of the NAA standard emerges (approximately four minutes, shown in Fig. 13). Then 200ul aliquots of sample were run in triplicate. Figures 14 and 15 show that the mean concentration of NAA was elevated in the running group vs the sedentary group (2.57 nmol/mg ± 1.02 for an n=6 and 0.58nmol/mg ± 0.15 for an n=7), $P \leq 0.05$.

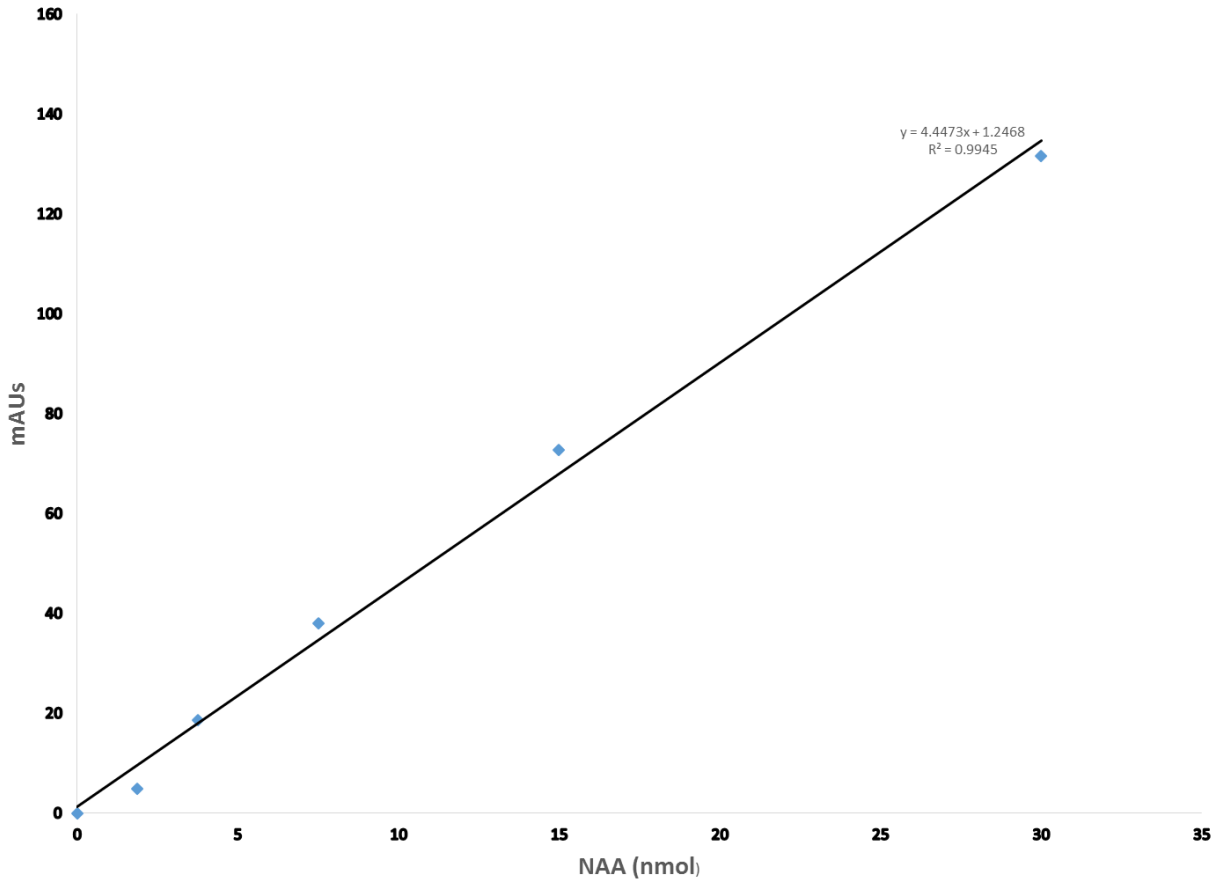


Figure 13. Standard curve. HPLC peak areas in absorbance units (mAUs) are a linear function of the amount of NAA (nmol) showing that NAA measurements were made within the linear range. Calculations were automatically performed using the associated software (R^2 = 0.99).

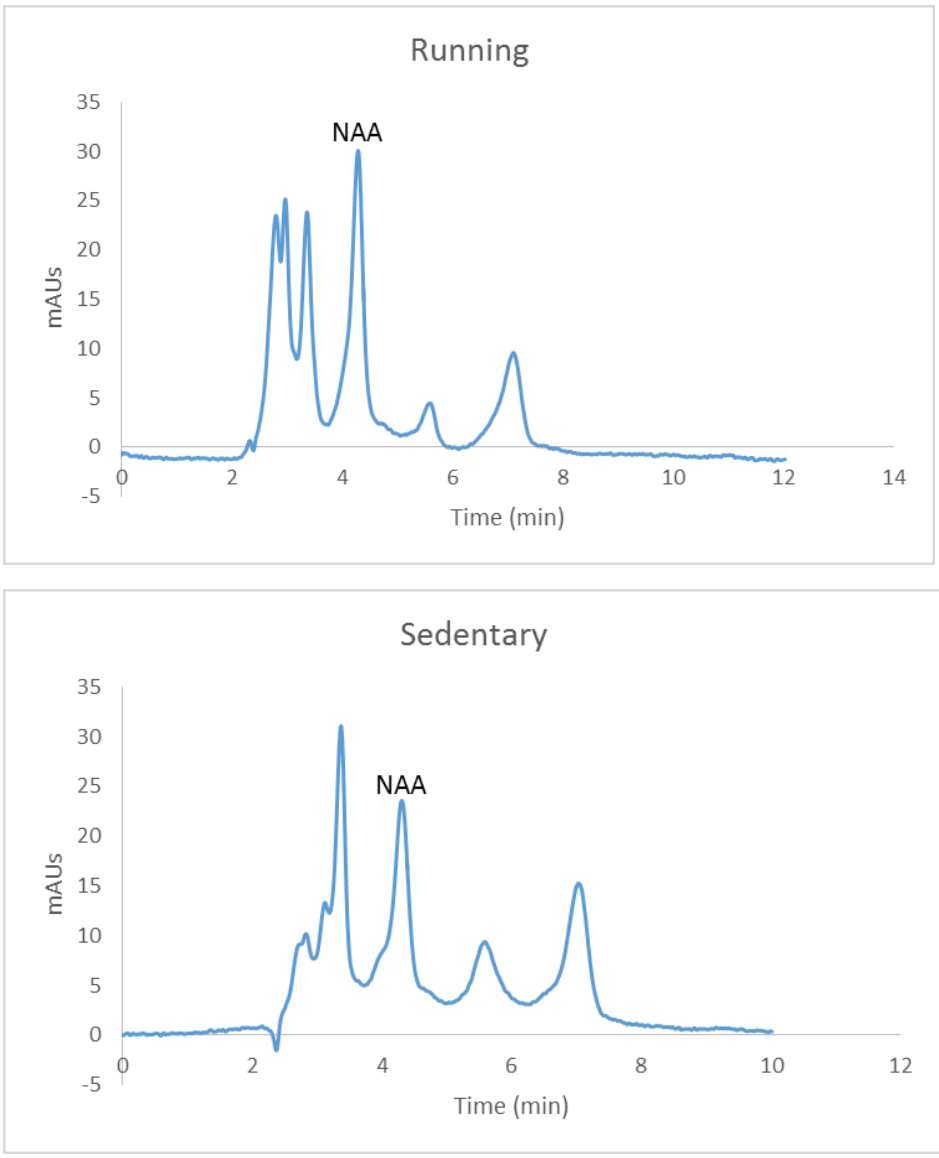


Figure 14. Representative HPLC chromatograms showing the presence of the NAA peak eluting at about 4 minutes in cortical samples obtained from running and sedentary animals.

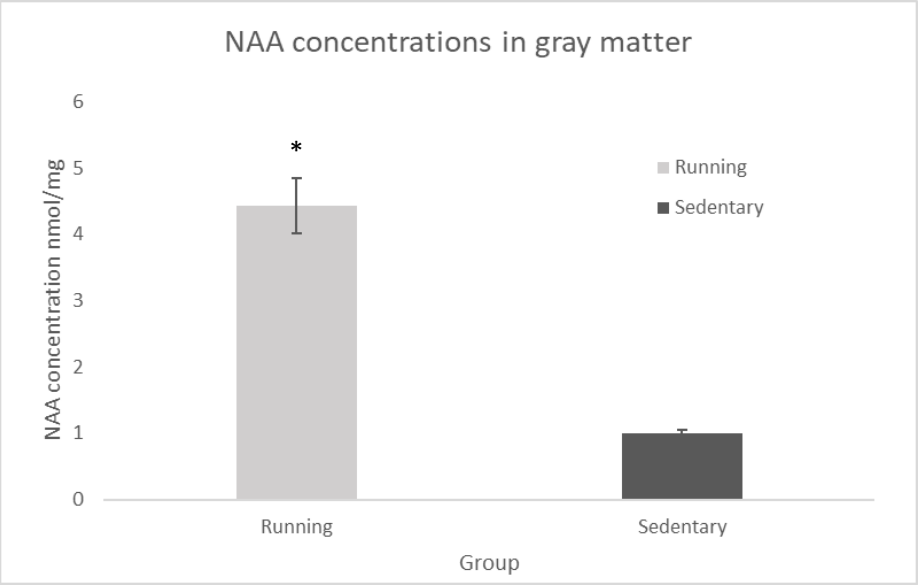
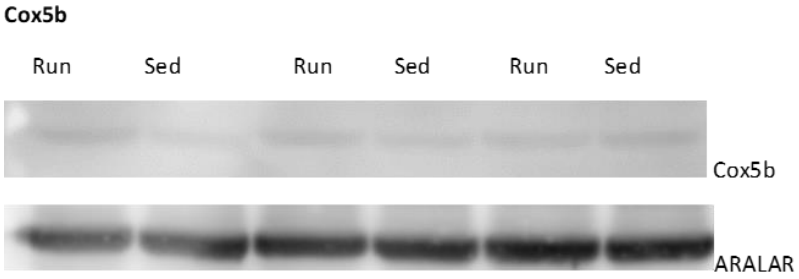


Figure 15. NAA is increased in gray matter of running rats. HPLC for NAA shows significantly higher concentrations of NAA in cortex taken from running animals vs. sedentary. Running animals showed a 4.4-fold increase over sedentary animals (n=6 and n=7, respectively), * P ≤0.05.

Cox5b expression in running animals

Cox5b is a subunit of cytochrome C oxidase (COX), the final complex in the electron transport chain. Cox5b is of particular relevance to our study because it is nuclear encoded, thus making it possible for nuclear-localized Hbb to influence its expression. Neurodegenerative diseases often correlate with changes in Cox5b expression, which lowers the efficiency of aerobic respiration in the brain. Figure 16 shows the results of densitometry for western blot with Cox5b in mitochondrial protein from rat cortex. These data show that Cox5b expression wasn't changed with running.

A



B

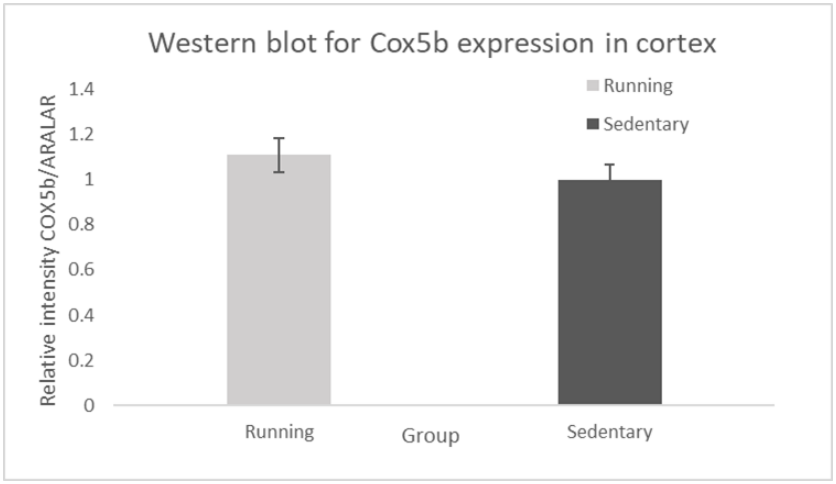


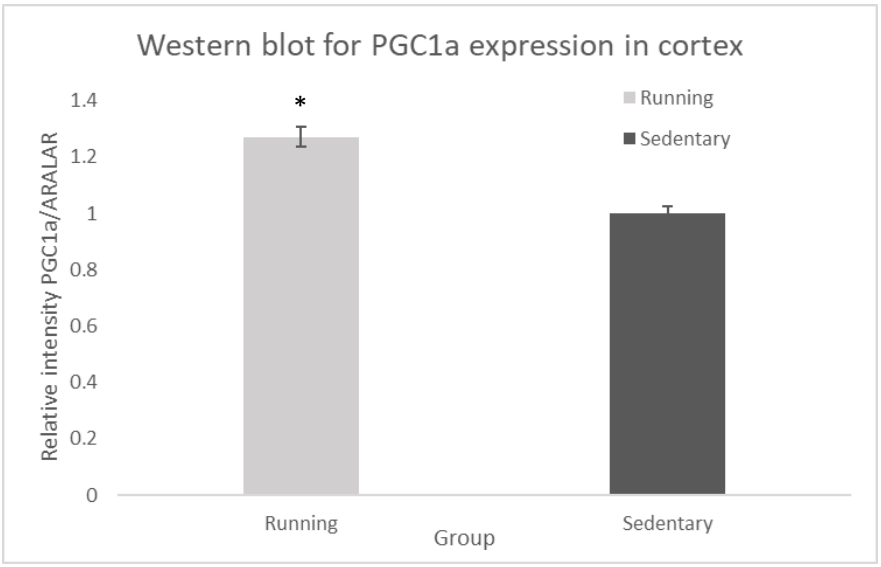
Fig. 16. COX B expression isn't changed with running. A. shows an example blot probed with a Cox5b antibody (Cox5b mus 1:100 in 5% BSA/TBST overnight) and re-probed using ARALAR.

B. The mean density for the running group was 1.86 ± 0.21 and 1.67 ± 0.19 for the sedentary group, (n=8).

PGC1α expression in running animals

Next, we examined changes in PGC1α, a marker of mitochondrial biogenesis that is crucial to preserving cell metabolic health during distress. Figure 17 below shows a 1.3-fold change in relative intensity in our running group over the sedentary group. We found that the average mean density for the running group was 1.16 ± 0.08 and 0.92 ± 0.064, $P \leq 0.05$. Fig. 17 also shows an example blot using PGC1α at 1:500 concentration in 5% BSA/TBST.

A



PGC1a

B

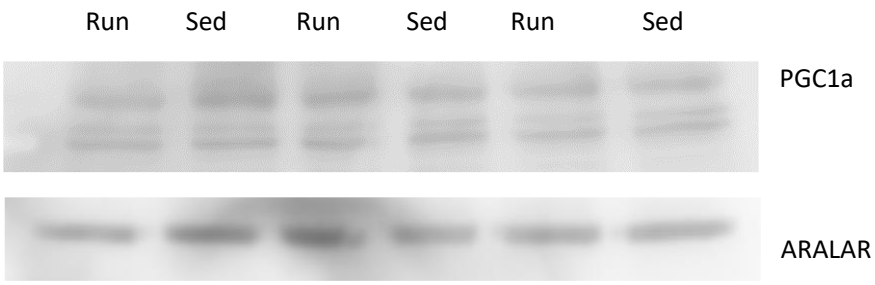
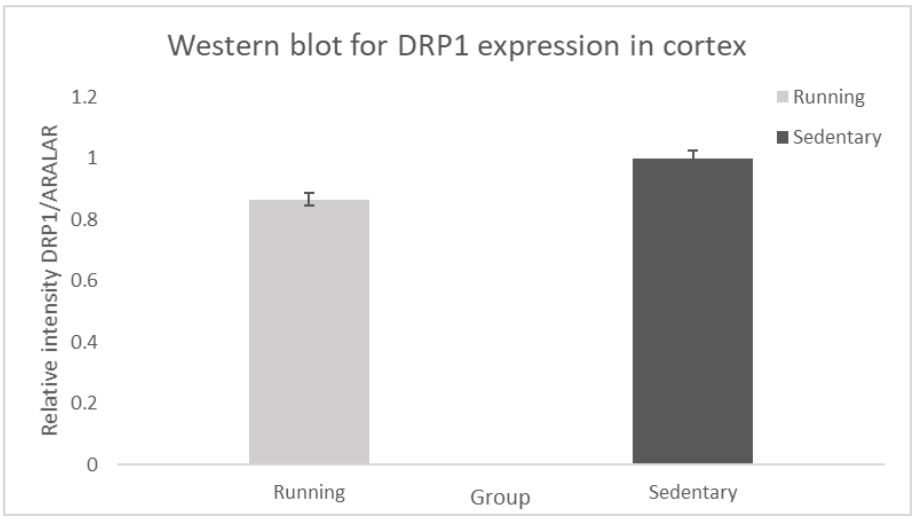


Figure 17. Western blotting shows that mitochondrial biogenesis factor PGC1α is increased in brains of running rats. A. We found that the average mean density for the running group was higher than sedentary, 1.16 ± 0.08 and 0.92 ± 0.064 (n=6), * P ≤0.05. B (bottom) shows a representative blot for PGC1α and ARALAR.

DRP1 expression running animals

Because changes in mitochondrial size and mitochondrial fragmentation occur in neurodegenerative disease states, we also looked at the mitochondrial fission protein DRP1 in running vs. sedentary animals. DRP1 expression may increase in order to clear damaged mitochondria and increase the number of mitochondria when needed. DRP1 increases can be a compensatory mechanism in diseases such as Alzheimer's and may be an indicator of metabolic health. With western blot (shown in Figure 18A), we found that DRP1 tended to be decreased in running animals. There was an average mean density of 0.69 ± 0.06 in running animals and 0.82 ± 0.06 for the sedentary group, a 1.2-fold difference over the running group, however, this change didn't reach statistical significance.

A



B

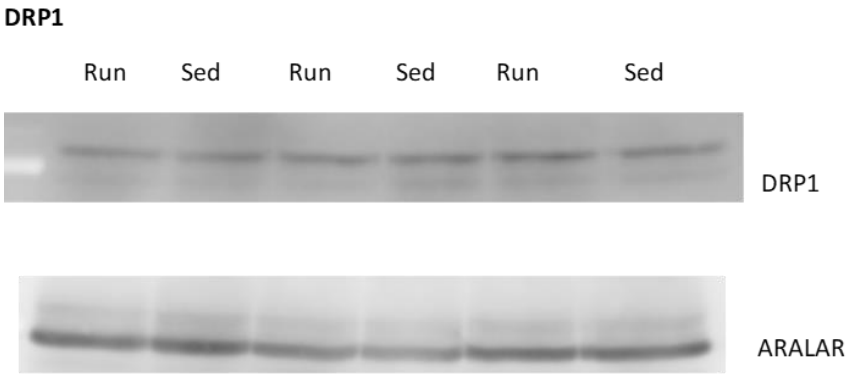


Figure 18. Effects of running on DRP1 expression. The mitochondrial fission protein DRP1 is unchanged in running rats. A (top) shows relative intensity of DRP1 in running animals, normalized to ARALAR. There was an average mean density of 0.69 ± 0.06 in running animals and 0.82 ± 0.06 for the sedentary group (n=7), but this difference was not significant. B (bottom) shows representative blot of DRP1 and ARALAR.

Rat TNFα ELISA assay results

For both trials using a total of 25 samples of protein, the assay produced no detectable quantities of TNFα in either group. Although the standard curves produced a low R2 value, making the results questionable (Figure 19 shows example), it is not uncommon for TNFα to be undetectable in tissues.

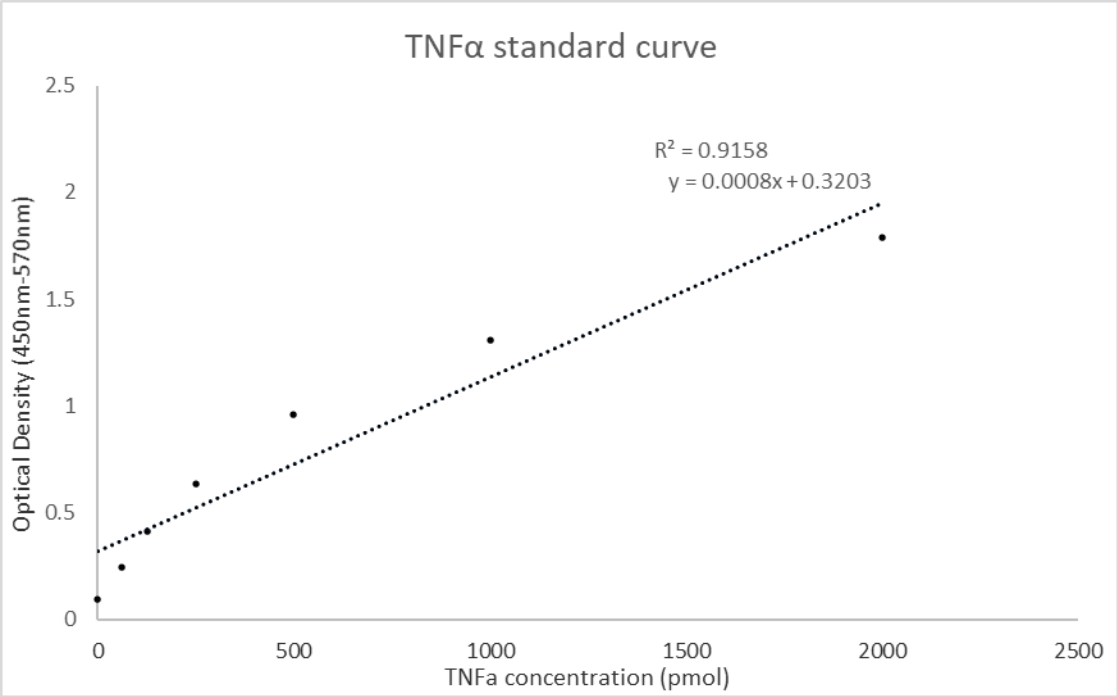


Figure 19. TNFa standard curve. The standard curve for TNFα using a subtractive method to remove values at 570nm as indicated by the supplier.

Seahorse Respirometry

We then looked at respiratory capacity in brain tissue from running and sedentary animals with a Seahorse respirometer to see if these the increases in the mitochondrial biogenesis factor

PGC1α (Fig. 17) correlated to increased mitochondrial efficiency in the running group. Figure 20 below shows that mitochondrial respiration as measured by oxygen consumption rates (OCR) over time was increased in the running group by approximately 50% for up to 75 minutes. P≤0.05 using a t-test.

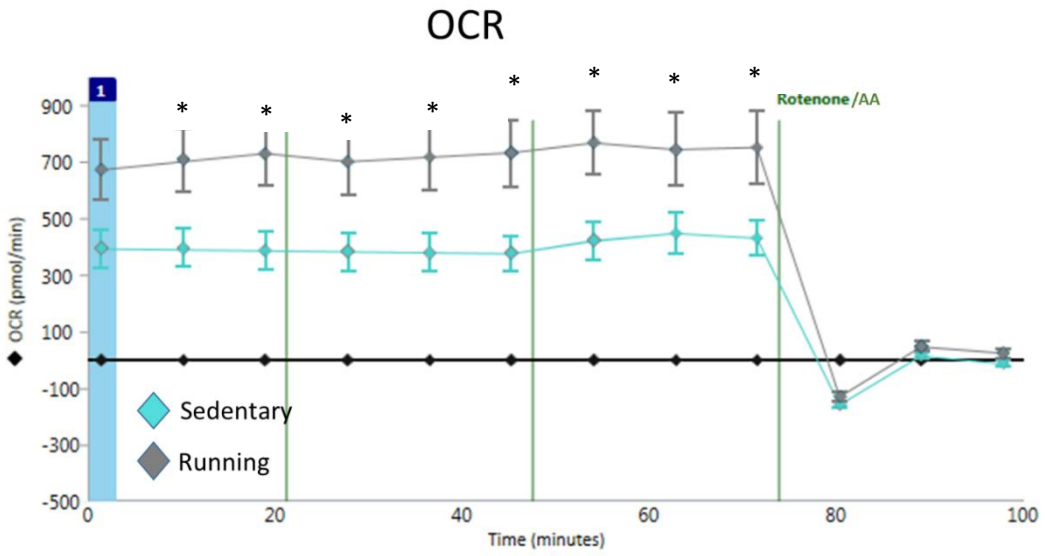


Figure 20. Oxygen consumption rate (OCR) in mitochondria isolated from rat cortex. Basal mitochondrial OCR in mitochondria isolated from cortex of running rats (n = 4) was increased compared to sedentary (n = 5) controls. OCR was measured by seahorse XFe24 analyzer. Cortical mitochondria have higher OCR when isolated from rats that have undergone 7 weeks of running (gray) when compared with sedentary control (green). The injections are 1µM antimycin A (AA) with 1µM rotenone during XFe24 measurements. Values between 0-75min were significantly different, student's t-test * P≤0.05.

Conclusion

Because of the close association between nuclear-localized Hbb and mitochondrial respiratory capacity seen in previous studies, we looked at basal respiratory capacity and the expression of nuclear factors associated with mitochondrial health, including the fission protein DRP1, mitochondrial biogenesis factor PGC1α and the nuclear encoded subunit of Cytochrome C oxidase, Cox5b. We found that basal respiration was significantly higher in our running group, and there was a significant increase in PGC1α. There was also a slight trend for Cox5b to increase in running animals. Surprisingly, the trend was for DRP1 to decrease in the running group, but this may be because increases in other factors required for mitochondrial efficiency were sufficient to affect metabolism. Furthermore, we saw a significant increase in NAA in the running group. NAA is a marker of mitochondrial efficiency, especially within the brain as it can serve as an important substrate for myelin lipid formation. Taken together, we can conclude that physical activity is capable of improving metabolism within the cortex.

Chapter 4

Exercise increases Hbb and EPOR in neurons and regulates expression of mitochondrial genes

Background

The hormone erythropoietin (EPO) was discovered to be responsible for increases in red blood cell (RBC) production by Francois-Gilbert Viault in 1890 through observation of individuals living at high altitude (Jelkmann 2011). EPO acts in response to reduced molecular oxygen availability in tissues. Hypoxia inducible factor proteins sense local levels of oxygen and interact with the EPO gene enhancer to promote its production, which is normally inhibited by GATA-2 repressor in normoxic conditions (Jelkmann 2011). Decreased oxygen availability occurs during moderately intense exercise, which is the reason why athletes tend to produce more RBCs. However, recent research on EPO has also indicated that in addition to regulating RBC production, it acts on other cell types to attenuate immune response and promote cell survival. EPO receptor (EPOR) is found widely in the brain including neurons, oligodendrocytes, astrocytes and microglia. EPO receptors are dimeric transmembrane receptors that bind and internalize single molecules of EPO, activating JAK-STAT pathways within the cell and inhibiting apoptosis (Ott, C. et al. 2015). A study on EPO treatment in the multiple sclerosis model experimental autoimmune encephalomyelitis showed a reduction in inflammatory markers including reduced glial activation and lower levels of TNFα (Agnello et al 2002). Similar studies in our laboratory have shown that treatment of cuprizone mice with EPO upregulates hemoglobin, increases NAA and increases H3K4me3 expression in the brain. The mechanism for this is unknown, but based on studies showing an association between Hbb expression in the nucleus and H3K4me3 levels, it is thought that Hbb plays a role in the methylation status of H3

and thereby affects expression of genes involved in cell survival and metabolism. Research from our laboratory on methionine metabolism and mitochondrial integrity has shown that treatment with the methyl donor betaine can enrich H3K4me3 at the promoter sites of the NRF1 and ERRγ genes. NRF1 is directly implicated in mitochondrial improvements via EPO signaling. NRF1 regulates mitochondrial biogenesis and antioxidant gene expression. A chip seq study for H3k4me3 following EPO treatment showed enrichment at the NRF1 promoter region, suggesting that EPO regulates mitochondrial health in part through the expression of this gene (Sollinger, C. et al. 2017). ERRγ is a member of the estrogen receptor family that has roles in multiple cell types. Upregulation of ERRγ is seen in hypoxic conditions, with some evidence to suggest that PGC1α regulates its increase. ERRγ functions to promote production of PDK4, a factor that slows the rate at which pyruvate is converted to acetyl-coA in order to maintain glucose homeostasis in low oxygen conditions (Lee J. et al. 2012).

Materials and Methods

Immunohistochemistry for EPOR

To visualize Hbb immunoreactivity, tissue sections were incubated in mouse anti-EPOR (1:100; mouse monoclonal) (Sigma) and chicken anti-NeuN (1:500) (Millipore Bioscience Research Reagents). All incubations were done in PBS, 1.0% Triton X-100, and 3% donkey serum overnight at 23°C. Tissue sections were then incubated in donkey anti-mouse Alexa-555 (1:1000) and donkey anti-chicken Alexa-488 (1:1000) for 2 h at 23°C. Both secondary antibodies were purchased from Invitrogen. Sections were washed and mounted with Vectashield mounting medium containing DAPI to label nuclei. Images were acquired with an Olympus

Fv1000 confocal microscope equipped with five laser lines (HeCd 442 nm, Ar 488 and 514 nm, HeNe 543 nm and HeNe 633 nm). Images were viewed with ImageJ (National Institutes of Health) and channels merged to show colocalized signals. Relative fluorescence was compared in running and sedentary tissue blocks. Images were captured sequentially for each channel to prevent bleed through and spanned the sections. An image mask was created using the NeuN channel as a guide to include the entire nucleus. The thresholded image mask was then used to clip the Hbb channel, and the pixels within the unclipped region were summed. This technique measures the amount of Hbb fluorescence from within individual NeuN-stained nuclei. Mean density of Hbb immunofluorescence was obtained from the average intensity from 6 NeuN-positive neuronal nuclei from at least 3 different images per sample.

qRT-PCR for ERRγ and NRF1

mRNA expression for the NRF1 and ERRγ genes was determined from 200mg of rat cortical tissue homogenized on ice using 1mL of TRIzol reagent per 100mgs. Samples were allowed to incubate at room temperature for five minutes following homogenization, and then they were spun down at 5000rpm for 5 minutes to remove debris. Supernatant was removed to fresh tubes at 23°C, and 0.2 parts of chloroform was added to each before vortexing for 15 seconds. The samples were allowed to sit for 20-30 minutes, and then they were centrifuged again at 10,000rpm for 10 minutes. The aqueous phase was carefully removed and placed in a new tube before adding 0.5 parts isopropyl alcohol. Samples were incubated for 10 minutes 23°C before another centrifugation at 10,000rpm for 10 minutes. Following this step, the isopropyl was removed and the RNA pellet was washed twice with 75% ethanol, vortexed and spun down. The final RNA pellet was allowed to dry completely before the addition of a minimal volume of

nuclease free water. RNA purity and concentration was determined using a biotek nanodrop machine. Samples with purity of 1.6 or higher were used for rt-qPCR. Rat primers for ERRγ (forward "GTC AGC AAT TGG AGC GGG A", reverse "ATT GAT GAA CCA GTA AAT TGT CAG C") and NRF1 (forward "GTA ACC CTG ATG GCA CTG TCT", reverse "CTC TGA TGC TTG CGT CGC T") were ordered from IDT DNA. Using a SYBR green ultra-fast master mix kit, the primers were added at a final concentration of 500nm each, forward and reverse. qRT-PCR was run in duplicate from RNA isolated from cortex from 4-6 animals per group. The master mix consisted of 10ul 2x SYBR, 0.1ul of each primer, 1ul RT and 0.2ul DTT per reaction run. To the master mix was added enough nuclease free water to bring each final reaction to 20ul. 19ul of this mixture was added to 10ng of RNA in 1ul. Quantitation was done with the $2^{-\Delta\Delta Ct}$ method with actin primers for normalization.

Western blot for H3k4me3

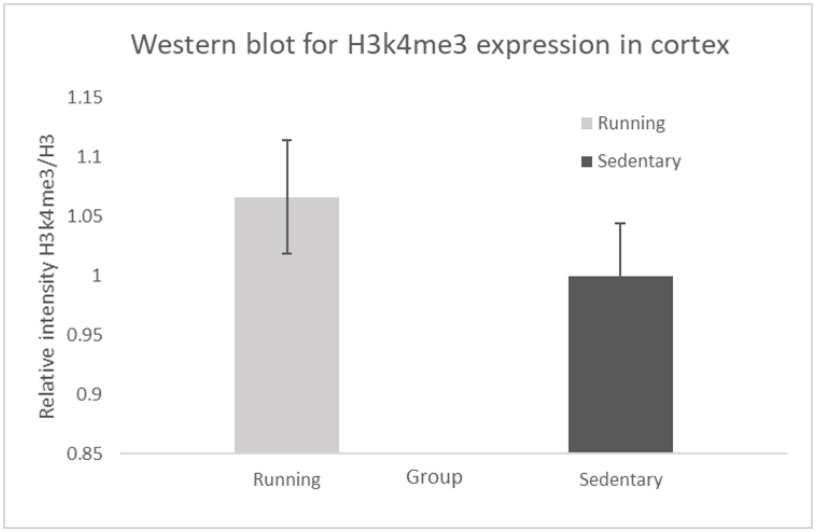
50ug of nuclear fraction protein was loaded on to two 15% gels to be run side by side for comparison using our standard electrophoresis and transfer protocols. Blots were incubated with either H3k4me3 rabbit (abcam) or histone H3 mouse at 1:500 concentration in 5% BSA/TBST overnight at 4°C. Blots were washed and secondary antibodies were applied according to our standard lab protocols. Data are from at least 8 animals per group (running and sedentary). We ran two side by side gels for H3k4me3 and H3 as a reference simultaneously, and blots were incubated overnight in H3k4me3 rb (1:500) in 5% BSA/TBST and H3 rb (1:500) at 4°C.

Results

H3k4me3 expression in running animals

Histone methylation status can often indicate a change in health, and H3K4me3 is a marker often associated with transcriptional activation that we have seen to be raised in some of our experiments under conditions that reversed mitochondrial dysfunction. Figure 21A shows that running animals tended to have a higher expression of H3K4me3 (1.152 ± 0.037) compared to the sedentary group (1.076 ± 0.023). However, the difference was not found to be significant (P-value of 0.08).

A



H3K4me3

B

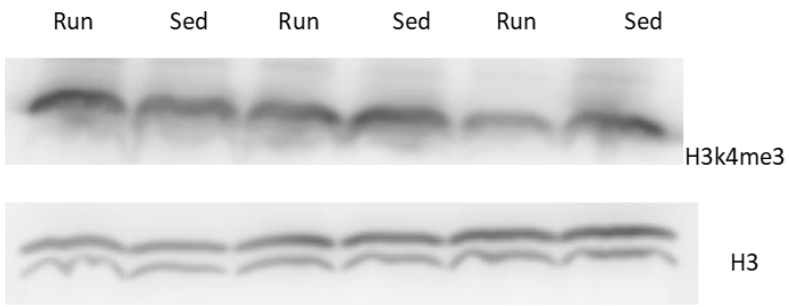


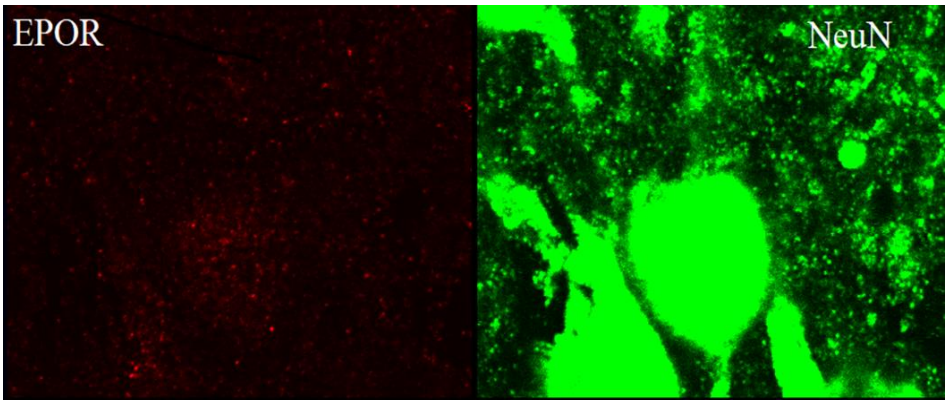
Figure 21. H3K4me3 is unchanged in cortex of running animals. A. (top)shows the difference in relative intensity of H3k4me3 in cortical tissues from running vs sedentary animals, normalized to the nuclear marker H3 (relative intensity values were 1.152 ± 0.037 and 1.076 ± 0.023, respectively for n=8). There was no significant change in H3K4me3 (p=0.08). B (bottom) shows representative blots probed for H3k4me3 and its respective marker, H3.

Erythropoietin receptor expression in cortical neurons

We have previously shown that activation of the brain EPO receptor (EPOR) can raise H3k4me3 levels and improve mitochondrial respiratory capacity. For this reason, we looked at changes in expression levels of EPOR in our running animals by immunohistochemistry. Using ImageJ to measure the mean density, we found that EPOR was indeed expressed at higher levels in the running group (0.269±0.028) when compared to the sedentary group (0.169 ± 0.029). This difference was found to be significant to a $P \leq 0.05$ using a t-test, as shown in Figure 22 and 23.

EPOR in neuronal nuclei of running animals

A



EPOR in neuronal nuclei of sedentary animals

B

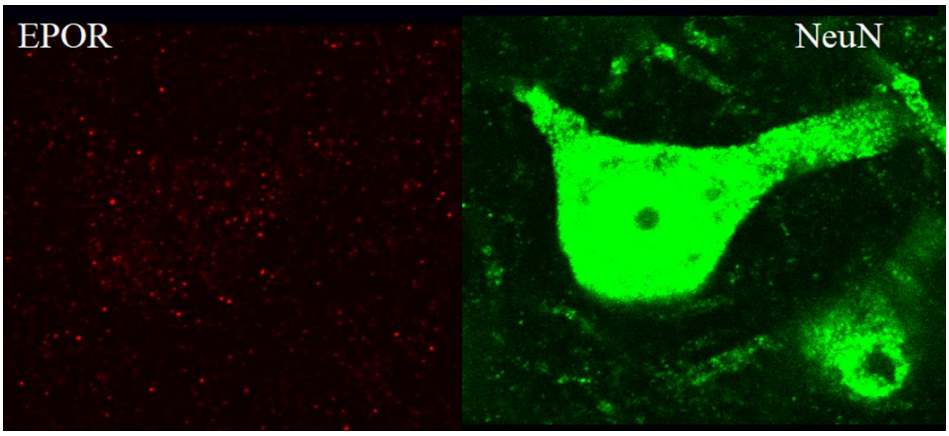


Figure 22. EPOR immunohistochemistry. Figures 22A and 22B show punctate staining for EPOR (red) with the neuronal marker NeuN (green) at 60x in 100um slices from the mid-frontal region in running and sedentary animals, respectively. EPOR was visibly more concentrated in the cortical neurons of running animals.

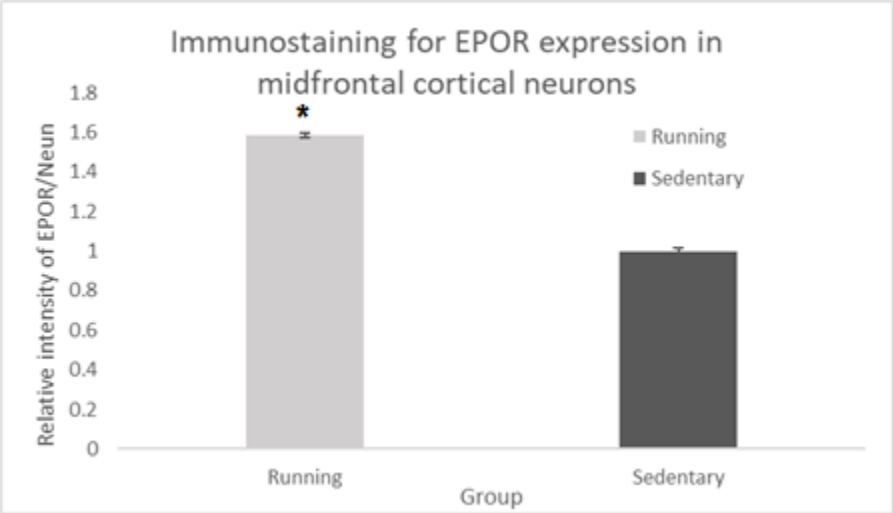
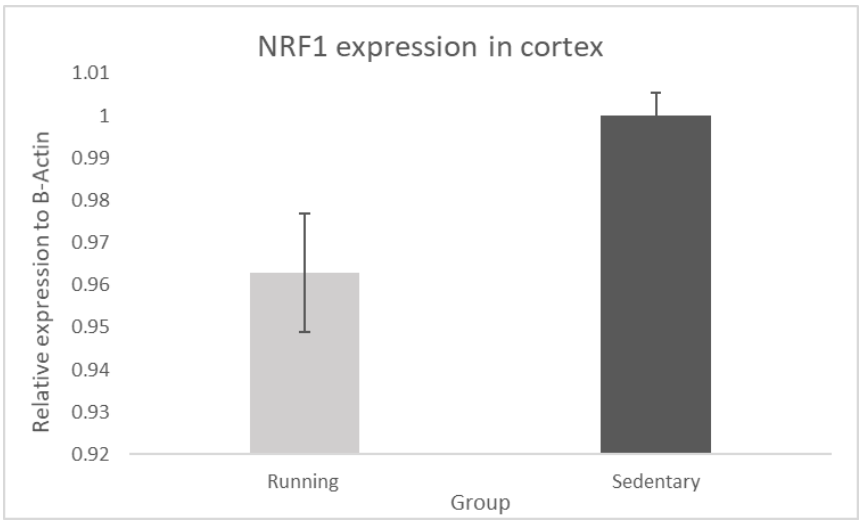


Figure 23. Quantitation of EPOR expression in response to running. Data show the relative intensity of fluorescence for EPOR over NeuN, normalized and corrected for area (running 0.269 ± 0.028 and sedentary 0.169 ± 0.029, n=5). Expression of EPOR was significantly higher in the running group, * P≤0.05.

Expression of genes associated with EPOR pathways

Previous research in our lab has shown that H3k4me3 methylation status influences the expression of two genes associated with EPOR pathways using chip seq. These genes were NRF1 and ERRy, nuclear proteins that are known to be involved in mitochondrial metabolism. We observed that the running group had a much greater degree of variation in H3k4me3 levels compared to the sedentary group, indicating that there may be changes in methylation patterns at sites relevant to metabolic health and cell survival. qrt-PCR revealed a decrease in the expression of NRF1 and ERRy in the running group. (Fig. 24A) There was a trend for NRF1 higher in sedentary animals (1.53±0.01) vs. running (1.47± 0.04). (Fig. 24B) ERRy was also higher in sedentary animals (1.53±0.03) vs. running animals (1.42± 0.03), $P \leq 0.05$. Expression changes in each sample were expressed as a ratio to beta-actin.

A



B

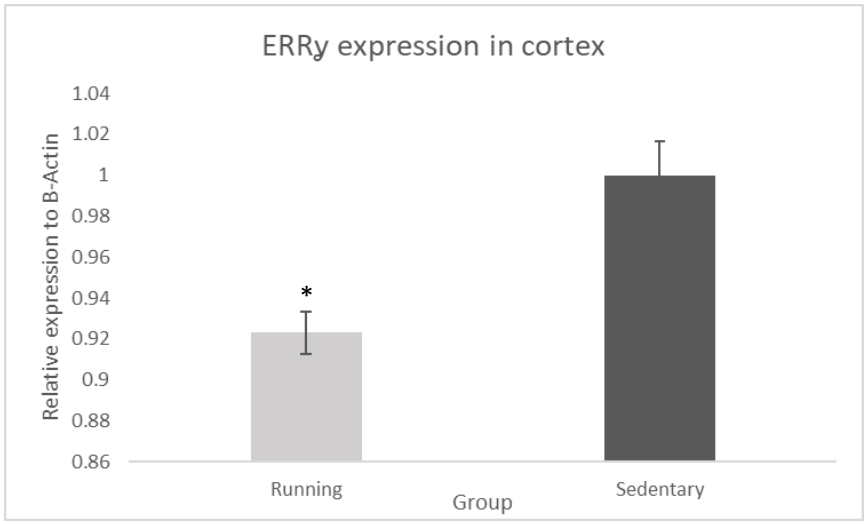


Figure 24. Expression changes in NRF1 (A, top) and ERRy (B, bottom) from rat cortical tissue. The expression of NRF1 tended to be lower in running animals (running 1.47± 0.04 n=6, vs sedentary 1.53±0.01 n=4), and we found significantly lower ERRy expression in the running group (running 1.42± 0.03 n=6, vs. sedentary 1.53±0.03 n=4, * P≤0.05).

Conclusions

We found that EPOR was significantly upregulated in the running group, indicating that exercise activates EPO-associated pathways in brain tissue. However, we did not find reason to link these changes to H3k4me3 levels, which showed only a slight trend to increase in the running group. For this reason, we chose to look at NRF1 and ERRγ, two genes identified in association with EPO-induced H3k4me3 methylation changes in previous studies. We found a trend for NRF1 to decrease as well as a significant decrease in ERRγ. These data suggest that the pathways stimulated through exercise reduce the need for the transcription of other genes associated with mitochondrial activity.

Chapter 5

Discussion

In multiple sclerosis, loss of myelin and degeneration of neurons and axons accumulate over the course of the disease and underlie the progressive disability associated with the disease (Bjartmar, et al. 2001; DeStefano et al. 2001). In the RRMS phase of the disease the autoimmune attack and inflammatory environment cause the degradation of myelin resulting in disruption of normal conduction of nerve impulses and neurological impairments, which under many circumstances resolves over a period of weeks. This resolution has been shown to result from a redistribution of sodium channels and remyelination (England, et al. 1990). As was indicated previously, neurons require significant amounts of energy as demanded by their high level of respiration. This requirement makes neurons particular vulnerable to disruptions in energy availability, particularly when they have experienced demyelination and redistribution of sodium channels along axons. In fact, it has been reported that altered neuronal energetics results in disruptions in ion homeostasis, conduction abnormalities and defects in axonal transport, all of which have been linked to neuronal death (Stys, et al. 2005; Waxman, et al. 2006). As a result of these repeated insults during the RRMS phase, neuronal and axonal damage accumulates leading to permanent and progressive disability. Subsequent progression to the SPMS stage is further associated with additional neuronal and axonal damage and ever-increasing disability. The progressive nature and impact of the neuronal and axonal damage underscores the need for identification of targets for neuroprotection (Confavreux and Vukusic 2006). Mechanisms responsible for the disruption in neuronal energetics involve alterations in mitochondrial function, including reductions in oxidative phosphorylation and changes in the

production of reactive oxygen species. Interestingly, these also appear to be involved in multiple neurodegenerative diseases including AD and PD (Norat, P. et al. 2020). Mitochondrial involvement in neurodegenerative processes is supported by earlier studies demonstrating a decreased expression of nuclear-encoded mitochondrial electron transport genes in non-lesioned gray matter and neurons in MS (Dutta, et al. 2006). This was also correlated with reductions in mitochondrial respiration in MS cortex (Dutta et al. 2006; Pandit et al. 2009; Broadwater et al. 2011; Witte et al. 2013). These reductions in mitochondrial activity have been suggested to contribute to neuronal pathology in MS and other neurodegenerative diseases by disrupting signal conduction and axonal transport (Waxman, et al. 2006). Thus, approaches to identify mechanisms capable of impacting neuronal mitochondrial function and restoring neuronal energetics would appear to be interesting targets for neuroprotective therapies. One such approach could involve a role for hemoglobin expression, since it has been demonstrated that in mice treated with EPO show increases in not only peripheral hemoglobin but in the brain as well (Horng, et al. 2015). Further, changes in hemoglobin expression in neurons has been shown to impact the expression of genes involved in mitochondrial respiration (Biagoli, et al. 2009) supporting the possibility that improved mitochondrial respiration and neuroprotection can be through pathways that impact neuronal hemoglobin expression. Thus, we explored the impact of physical exercise on several of these pathways. Here we found that rats (8-10 weeks to start) provided with free access to running wheels will run on average nearly 3k/night for a span of seven weeks. This level of activity is consistent with previous reports demonstrating that rats between the ages of 12-24 weeks ran between 2-3k/night (Judge, et al. 2005) and previous studies have demonstrated that 7 weeks of running

were also linked to neurogenesis (Nokia et al. 2016). Running as well as other exercise regimens have been linked to multiple beneficial effects related to brain function. In both humans and animals, physical activity targeted to impact cardiovascular/pulmonary fitness has been linked to improvements not only in improved delivery of nutrients and oxygen to all tissues through enhanced physiologic performance but also through multiple other mechanisms. These include enhanced vascularization in brain, improved glucose handling and production of important growth factors, including BDNF, that impact generation of new neurons and synapses (Cotman et al. 2007; Mattson 2012; Wrann et al. 2013 Morland, C. et al 2017; Erikson, K. et al 2012). Further both growth hormone (GH) and insulin-like growth factor (IGF-1) are increased with physical activity as well (Frystyk 2010) and both have been shown to positively influence hemoglobin expression in the brain (Walser et al. 2020). Consistent with this, our findings demonstrate that physical exercise in rats supports the increased expression of hemoglobin in neurons.

Voluntary wheel running increases hemoglobin beta in neurons

Based on studies of MS in post-mortem tissues, we know that nuclear localization of hemoglobin beta can influence the expression of vital mitochondrial genes that are involved in the interplay between metabolism and inflammation. We posited that increases in nuclear expression of hemoglobin beta would occur with the addition of a voluntary wheel running regimen in our rats. Western blot and immunohistochemistry results suggest that voluntary exercise does increase hemoglobin beta in neurons. This increase in expression, while not significant (P=0.06), appeared to be consistent in the nuclear fraction of running vs. sedentary

animals. This change was, however, confirmed with immunohistochemistry, where we were able to document a significantly higher amount of hemoglobin beta localized to neuronal nuclei in animals exposed to seven weeks of voluntary running. Here there was a significant increase in the staining for Hbb and this staining appeared to be localized to nuclei in neurons. Animals provided access to running wheels showed an increase in Hbb expression of 80% and 50% respectively. Expression of Hbb in the neurons is not unique, our group and others have shown that hemoglobin is expressed in neurons in the rodent and human brain (Biagoli et al., 2009; Richter et al., 2009; Schelshorn et al., 2009; Broadwater et al., 2011; Brown et al., 2016). The regulation of hemoglobin in the brain is closely linked to metabolism, and there is emerging evidence that it may function in the regulation of mechanisms that impact gene transcription and not solely as an oxygen carrier. Hemoglobin α and β subunit (Hba and Hbb) mRNAs as well as proteins have been found to be expressed in neurons in the cortex, substantia nigra, and hippocampus. We have found that both Hba and Hbb are localized to the cytoplasm in primary neuronal cultures but only the Hbb subunit was relevant to changes seen in MS (Singhal et al. 2018). We previously performed co-IP followed by LC-MS/MS to identify Hbb interacting proteins in total cell extracts. We found that Hbb interacts with both mitochondrial and nuclear proteins including histones in the human brain and in rat primary neurons (Brown et al., 2016). Thus, Hbb has been identified in brains and neurons previously and its expression can be modulated under several physiologic conditions, including cellular stress, EPO signaling and hypoxia (Richter et al 2009, Biogoli et al. 2009, Shelshorn et al. 2009, Singhal et al. 2018). Further changes in Hbb expression in these brains in response to running could also be the result of several factors that are induced by physical activity, including release of growth

factors, including GH, IGF-1 (Walser et al. 2020) and even BDNF (Knaepen et al. 2010), all of which have been shown to be increased as a result of physical activity. This may be of significance under pathological conditions since, changes in neuronal hemoglobin expression or subcellular localization have been linked to multiple sclerosis (MS), Alzheimer's disease (AD), and Parkinson's Disease (PD)(Brown et al., 2016; Ferrar et al., 2012; Shephard et al., 2017) and each of these neurodegenerative diseases exhibit a dysfunction of mitochondria that contributes to disease pathology. Hemoglobins and oxygen regulation within the cell are inextricable. Activation of hypoxia inducible factors in low oxygen conditions is a signal to the cell to increase activity in the erythropoietin pathway and upregulation of hemoglobin expression. It is likely that exercise affects Hbb levels because of an increased demand for ATP and thus an increase in oxygen utilization. Indeed, it has recently been reported that wheel running in mice creates local hypoxia in several brain regions and stimulates neurogenesis (Wakhloo et al. 2020). These local changes in hypoxia could serve as potent signals aimed at increasing mechanisms necessary to condition the cells to cope with the changes in functional demand. In particular, increases in Hbb in neuronal nuclei may play a role here and ultimately impact mitochondrial gene expression and respiration. What an exercise regimen would mean for studies with human beings is still unclear. But investigations into what level and type of exercise might be most beneficial in terms of its impact on neuronal Hbb expression would be interesting studies to examine.

Hemoglobin increases mitochondrial respiratory capacity

We know that Hbb is capable of interacting closely with promoter regions of several nuclear-encoded mitochondrial genes based on co-immunoprecipitation data from cultured rat primary cells (N. Brown. et al. 2016). Further, several lines of evidence suggest that not only does Hbb interact in these regions it impacts transcription as well. Overexpression of Hbb but not Hba can increase H3K4me3 levels which is linked to increased mitochondrial respiration in neuronal cell culture. In addition, studies have also demonstrated that treatment of cuprizone mice, a treatment that causes pronounced myelin degradation, with EPO (5000u/kg) results in increasing Hbb expression and improved mitochondrial respiration that is also associated with recovery of myelination. It was suggested that this mechanism is most likely through increases in the methylation of H3k4me3 (Singhal. et al. 2018). These increases in Hbb expression were also associated with increased expression of crucial subunits to the mitochondrial complexes III and V, increased basal respiration rate and increases in the marker of neuronal mitochondrial activity, NAA. Based on these previous findings and the fact that exercise is capable of inducing HIFs, as well as, EPO signaling pathways in the brain we anticipated that exercise may mediate similar changes in mitochondrial gene expression and respiration in our animals.

We anticipated that if hemoglobin beta acted on nuclear-encoded mitochondrial genes, as has been reported previously, that we would observe changes in respiratory capacity, expression of respiratory complex genes and possibly in mitochondrial biogenesis markers. Our running animals showed an increased basal oxygen consumption rate. We focused on the expression of Cox5b, since it has been shown to be decreased in MS and contains a nuclear encoded subunit. While there was a trend for Cox5b to be expressed higher in our running group, it was not

significant. This could also be a result of contamination from different cell types if the role we proposed for hemoglobin beta only applies in neurons. Interestingly, the mitochondrial biogenesis factor PGC-1α was increased in running animals, as would be expected from studies on PGC-1α and exercise in the periphery. It could be that PGC-1α expression is essential to exercise induced hemoglobin beta expression in nuclei, as its expression allows stabilization of hypoxia inducible factor 1α (HIF-1α), the HIF that controls hemoglobin transcription. This increase in PGC-1α expression would also be consistent with the increase in oxygen consumption we observed in mitochondria isolated from brain of running animals compared to sedentary controls. PGC-1α is linked with mitochondrial fusion which has been shown to enhance mitochondrial coupling efficiency and oxidative phosphorylation (Yao et al. 2019). The trend for DRP1 to be expressed at lower levels in running animals suggests that improvements in mitochondrial metabolism and biogenesis through exercise signal to the cell that fission and mitophagy are not needed as much. DRP1 expression has been proposed to play an important role in the disposal of damaged mitochondria and appears to be an important signal in apoptotic pathways as well (Lee et al. 2004). Thus, under conditions of improved mitochondrial respiration it might be expected that this signal would be down-regulated. In fact, increased expression can become detrimental to the cell, as is the case with some neurodegenerative diseases.

Within the cell, damage or loss of mitochondria can create a demand for the clearance of dysfunctional organelles and the production of new ones. Dynamin-related protein 1 (DRP1) is a GTPase-based mitochondrial fission regulator and mitophagy regulator (Itoh, K. et al. 2013). When mitochondrial dysfunction interferes with aerobic efficiency, fission of remaining

mitochondria can help to compensate. In these cases, DRP1 expression also helps to facilitate mitophagy and clear damaged mitochondria. DRP1 activation is regulated by serine phosphorylation by protein kinases. When DRP1 activity is not useful, Protein Kinase A phosphorylates serine637 to inactivate it. Many modifications can be used to regulate the activity of DRP1 including ubiquitination, somoylation and other types of phosphorylation. The GTPase activity of DRP1 is distinct from many of its other functions when modified, but its goal is to enhance mitophagy and fission. However, when DRP1 accumulates it can be a detriment to the cell. Raised levels of DRP1 have been associated with necrosis and inflammation in diseases such as Alzheimer's, ALS, Parkinson's and cerebral ischemia. (Itoh, K. et al. 2013; Choi, S.Y. et al. 2020; Flippo, K.H. et al. 2019). Additionally, there is a delicate balance between PGC-1α - mediated mitochondrial biogenesis and mitochondrial fission via DRP1. PGC-1α increases may lead to increased levels of DRP1 following an insult to the cell, but its knockdown can also lead to increases (Dabrowska, A. et al. 2015; Singh, S.P. et al. 2015). The latter relationship has been shown to lead to decreases in sirtuins and other proteins related to reduction of ROS. The decreasing trend of DRP1 expression in our running animals suggests that exercise is capable of enhancing aerobic respiration through a mechanism independent of mitochondrial fission and mitophagic processes. Increased expression of PGC-1α in the running group further suggests that DRP1 expression was decreased because mitochondrial biogenesis was enhanced with exercise. We have observed alterations in several additional respiratory complexes induced by hemoglobin and EPO, including ATP5a1 and ATP5B. Whether these subunits are affected by running is not known and would be important future studies to examine.

Exercise-induced signaling to support improved mitochondrial function

Our results suggest that mitochondrial basal oxygen consumption increased in the running group and was associated with increased expression of PGC-1α. PGC-1α stabilizes HIF-1α and is capable of assisting in activation of its associated pathways. HIF-1α is known to be the main HIF involved in increased transcription of hemoglobin beta. HIF-2α, on the other hand, is the main factor responsible for regulation of EPO receptor expression and EPO production. Therefore, we chose to look at EPOR expression in the cortex. Immunohistochemistry showed that EPOR was increased in neurons of the running group, likely indicating that EPOR signaling is responsible for some of the changes we saw in mitochondrial metabolism. Indeed, it has been reported recently that EPOR expression in neurons is increased in running mice and is linked to increased EPO production in these same neurons (Wakhloo et al. 2020). Based on prior research from our lab showing that hemoglobin beta expression can increase levels of the transcriptional activation marker H3k4me3 and expression of nuclear genes involved in mitochondrial metabolism, we sought to ascertain the likelihood that the benefits we saw in respiration were a result of H3K4me3 levels. While there was a trend ($p=0.08$), we did not see a significant difference in H3k4me3 expression between the running and sedentary groups using western blot. Because the levels of methylation are not always as important as overall methylation patterns, we looked at two candidate genes identified in previous chipseq studies of H3K4me3 from out lab, NRF1 and ERRγ. There was a trend for these transcripts to be decreased in the running group. This may be consistent with the findings related to expression of the electron transport chain subunits described above, since they too did not appear to be increased with running. NRF1 is an important transcription factor that has been shown to activate key

metabolic genes required for mitochondrial respiration including COX5B (Kiyama et al. 2018). Thus, the lack of changes in these nuclear encoded mitochondrial genes may the result of a lack of NRF1 signaling under these conditions. ERRγ an estrogen receptor family subtype involved in oxygen-dependent transcription of many genes and plays a key role in neuronal mitochondrial metabolism (Misra, et al. 2017). It is known that HIF-1α activity is capable of downregulating ERRγ, and may be responsible for the changes we observed in our running animals since it has been reported that running creates a hypoxic environment in the brain and has been suggested to induce HIF-1α expression and subsequent EPO production in neurons (Wakhloo et al. 2020). These findings may suggest that exercise, while it increases expression of both Hbb and PGC-1α, may not cause increased mitochondrial gene expression or biogenesis but rather increased mitochondrial fusion and improved respiration as observed in these studies.

Regulation of EPO production and hypoxia related pathways

What we know about EPO regulation in the cell is that hypoxia is the initial factor in upregulation of the receptor and EPO cytokine production. Hypoxia in the cell opposes the constitutive targeted destruction of hypoxia-inducible factor 2alpha (HIF-2α) by an enzyme known as prolyl-hydroxylase domain 2, or PHD2 (Lappin, T.R. & Lee, F.S. 2019). Once no longer modified for destruction, HIF-2α binds to its partner ARNT and stimulates transcription of EPO, which then binds to its membrane receptor and activates several pathways including the JAK-STAT pathway associated with survival signaling and the PI3K/Akt pathway which decreases the mitochondrial permeability transition and NfKβ levels through the inhibition of GSK3B (Bo, P. et al. 2020). This and MAPK activity stimulated by EPO binding its receptor serve to decrease

inflammation in the cell and oppose apoptotic signaling. Through EPO expression, GATA-3 and -4 are activated along the EPOR promotor region in neurons to upregulate its expression (Wallach, I. et al. 2009). Contradictory to our expectations, there was no clear relationship between exercise, H3K4me3 levels and the expression of EPOR in our study. While this was unexpected in the context of previous research from our laboratory, it has been shown that decreased methylation can also enhance EPOR expression in neurons (Wallach, I. et al. 2009). As for Hb production, the transcription of all subunits in neuronal cells is regulated by HIF-1α levels and heme availability (Saha, D. et al. 2014). Like HIF-2α, HIF-1α is expressed stably under normoxic conditions, but oxygen-dependent enzymes add a ubiquitin tag that targets it for degradation (Masoud, G.N. & Li, W. 2015). PI3K can also upregulate HIF-1α through Akt-dependent phosphorylation and disruption of eukaryotic initiation factor 4E (eIF4E) and its binding factor (4E-BPI), inhibitory initiation factors at its mRNA translation initiation cap. Somewhat paradoxically, SIRT1, a factor that is associated with mitochondrial health and cell survival, also downregulates expression of HIF-1α. In hypoxic conditions, suppression of SIRT1 by NAD+/NADH production during glycolysis allows HIF-1α transcription to increase. The mechanism for this increased transcription involves P300/CBP recruitment to the HIF-1α target genes by acetylation. However, histone deacetylase inhibitors have been shown to decrease HIF-1α levels and antagonize subsequent pathways. Although we did not look at HIF-1α or HIF-2α in this study, it can be assumed from the increased Hbb and EPOR that both of these pathways were involved. Exercise has been shown to alter both through hypoxia and production of ROS. In fact, studies on HIF-1α and HIF-2α in substantia nigra dopamine neurons have shown that voluntary wheel running produces a bell-curve in HIF-1α levels over time, with

the highest level of expression occurring at day 5 and returning to baseline by day 7 (Smeyne, M. et al. 2015). HIF-2α does not change significantly with exercise, but there is a trend for it to increase over a longer time scale before returning to baseline. Of interest here is whether or not the mechanisms of improved aerobic respiration in our animals were dependent on Hbb increases. Because we did not do a conditional knockout study with exercised animals, we cannot conclude what importance Hbb has in the adult neurons of the cortex. This means that we cannot conclude that Hbb is the driver of any of the differences between the running and sedentary animals in our study. However, it is known that in skeletal muscle, PGC-1α -mediated improvements in mitochondrial health depend of calcium signaling, insulin levels and AMP/ATP ratios, suggesting that its benefits can occur in the absence of factors in the HIF-1α /HIF-2α pathways (Fernandez-Marcos, P.J. & Auwerx, J. 2011). Importantly, PGC-1α can elevate levels of HIF-1α by stabilization of its levels in skeletal muscle. PGC-1α has its effects on mitochondrial biogenesis through NRF1/NRF2, ERRa, Gabpa, PPARs, and the thyroid hormone receptor. This in turn increases mitochondrial respiration and thereby increases oxygen consumption, leading to the stabilization of HIF-1α (O'Hagan, K.A. et al. 2009). Given its place at the head of all of the pathways we looked at in the course of our study, we can speculate that PGC-1α is necessary for Hbb and EPOR increases following exercise. Future studies might look for changes in sirtuins in running animals, and check cortical expression levels of PGC-1α with acute exercise. Part of the reason that exercise is a factor of interest in the study of inflammation is that it serves as a stressor. Common knowledge tells us that exercise poses a benefit to the body, but at a cellular level, the addition of exercise is a type of stress. This leads to activation of pathways that are central to many inflammatory diseases, however, paradoxically, exercise is equally capable of

decreasing activity along these pathways over time. It is for this reason that looking at the precise balance of targets critical to cellular metabolism and inflammation is more telling than the simple presence or absence of any one target.

PGC-1α, Sirt1 and NAA in mitochondrial dynamics

PGC-1α may indicate enhanced mitochondrial activity because of its role in mitochondrial biogenesis, but there are other good indicators of metabolic activity in brain tissue. N-acetylaspartate (NAA) is a compound found almost exclusively in gray matter of the brain. Its synthesis depends on aspartate and coenzyme A, a reaction that is carried out by the enzyme N-acetyltransferase (Amaral, A.I. 2017). NAA is a major source of acetate, a biomolecule that plays a signaling role in cells through g-proteins that affect cyclic amp, and in the form of acetyl-CoA, which may then influence signaling through direct acetylation (Moffett, J.R. 2020). NAA is released by neurons to oligodendrocytes, where it is crucial to myelin lipid synthesis. In addition, NAA regulates glutamine/glutamate oxidation and histone acetylation and aerobic respiration (Amaral, A.I. 2017). In general, NAA is associated with greater mitochondrial metabolism and lower levels of inflammation. Conversely, NAA is decreased in Alzheimer's MS and Schizophrenia. NAA declines naturally with age, but higher cardiovascular fitness level has been associated with less decline in NAA levels in older adults and better performance on working memory tasks (Erickson, K.I. 2012).

NAA is normally broken down into acetate and then converted to acetyl-CoA to be used in myelin sheath production by the enzyme ACSS2 (Moffet, J.R. 2020). The activity of this enzyme is enhanced by SIRT1, the sirtuin that also deacetylates PGC-1α and promotes its activity, making it an important factor in mitochondrial biogenesis (Tang, B.L. 2016). SIRT1a transcripts have been shown to increase within two hours following physical activity in humans, meaning that many of the changes we saw at the end of our study may have been initiated by SIRT1 increases, something future studies may take into account (Guerra, B. et al. 2010).

The two candidate genes we examined in this study are known to be under partial regulation of PGC-1α as well, and both contribute to mitochondrial biogenesis. The slightly lower expression of ERRγ in our running group was a surprise, since we know that ERRγ increases fatty acid oxidation, mitochondrial biogenesis and angiogenesis. In skeletal muscle, ERRγ is likely to be responsible for vascularization, making these results puzzling (Narkar, V.A. et al. 2011). However, it is possible that ERRγ was increased to compensate for reduced cerebral blood flow in the sedentary group. NRF1 is activated by PGC-1α to increase synthesis of mitochondrial DNA, which makes the lower levels seen in the running group quite paradoxical (Bereiter-Hahn, J., Jendrach, M. 2010). The trend might be explained by the role NRF1 plays in lipid oxidation, where it complexes with SIRT6 and FOXO3a to control transcription of genes involved in triglyceride synthesis. Loss of SIRT6 leads to fatty liver disease in mouse models of metabolic disease (Hassanieh, S. & Mostoslavsky, R. 2018).

Ceramides and neurodegeneration

Because the myelin sheath is composed of lipids, the lipid chemistry of the brain is arguably of even greater importance than it is elsewhere in the body. Ceramides are a class of lipid derived from breakdown of sphingomyelin by sphingomyelinases (SMASEs) from the cell membrane, or de novo synthesis by the ceramidase enzymes. They have various functions within the cell, depending on their length. Some of their signaling roles include senescence, motility, growth, adhesion and differentiation (Tong & Monte. 2015). Certain ceramides can initiate caspase cascades and cause mitochondrial distress when present in high amounts. C6 increases pro-apoptotic caspase 2, C16 aids in channel formation in the mitochondrial outer membrane and C18 inhibits Cytochrome C activity (Groves et al 2013; Qin et al 2010). These and several other ceramides are present in abnormal ratios in patients with Alzheimer's, Parkinson's, MS and ALS (Alessanko & Albi. 2020). Ceramides are potentially involved in the neurodegeneration witnessed in type II diabetes and obesity. In vitro studies of cells treated with C6 showed an increased expression of insulin and IGF receptors consistent with insulin resistance. It is possible that C6 ceramide crosses the blood-brain-barrier in metabolic disease and produces mild degeneration (Tong & Monte. 2015). Loss of lipid regulation is an important aspect of neurodegeneration in Alzheimer's, MS and PD. Changes in the ceramide ratio may occur as the result of a mutation in any of the enzymes involved in the production of ceramides or their precursors, as well as the enzymes responsible for breakdown. Cell culture experiments on Alzheimer's have shown that upregulation of nSMASE and production of ceramides in the presence of Aβ contributes to inflammation and cell death (Alessanko & Albi. 2020). Studies on Parkinson's show dysregulation of ceramides that correlate to specific types of cognitive issues,

demonstrating the unique roles of different ceramides and the importance of maintaining a certain ratio within the cell. The effects of ceramides on erythrocytes underlies a possible role for exercise in the maintenance of the ceramide balance within the cell. A study on erythropoiesis showed that C6 and C2 are produced by activation of TNFαr1 and prevent the expression of important lineage markers. EPO did not stimulate erythropoiesis in the presence of TNFα, which suggests that the ceramide ratio may be critical to the effectiveness of stimulating the EPO associated pathways in other cell types (Orsini, M. et al. 2019). This, along with the detrimental role that ceramides may play in metabolic disorders implies that exercise might be used to prevent dysregulation of the system. Our initial TLC results suggest that ceramides may have been slightly elevated in the sedentary group compared to the running animals. This is in accord with studies that have shown that exercise decreases levels of pro-inflammatory ceramides and associated enzymes over time in skeletal muscle, which may be a key factor in preventing insulin resistance (Bergman B.C. et al. 2016: Helge, J.W., et al. 2006). However, we did not obtain our HPLC-MS results for the respective ceramide ratios in each group before the conclusion of our study because of equipment difficulties and pandemic regulations. We expect to receive the data in the near future, and we are interested in knowing if the lipid composition might be related to the expression of EPOR and activity of anti-inflammatory pathways. A relationship between membrane sphingolipids and EPOR expression has been previously demonstrated by the importance of lipid rafts in receptor stabilization (K.L McGraw et al 2012), and the importance of TNFα-associated ceramide signaling in inflammation and apoptosis is well defined. In short, a better understanding of how exercise

might influence the lipid composition of cells within the brain could elucidate ways of preventing the loss of myelin and cells in neurodegenerative states.

Exercise as a preventative against neurodegeneration

Our data revealed that exercise is capable of producing metabolic improvements in adult Sprague Dawley rats that have no disease and no prior physical enrichment. Our voluntary wheel running design allowed for a wide range of activity levels in the experimental group, but despite this possible confound, we saw changes in many of the targets we chose to examine. For this reason, we see exercise as a promising avenue in for the prevention of disease, and possibly as an adjunct therapy for neurodegenerative diseases.

Exercise has long been touted as a preventative against inflammatory conditions such as heart disease, diabetes and obesity. Studies have shown benefits of a wide range of different types of physical activity in humans, from walking to weight training (Netz, Y. 2019). In recent years, the realization that the blood-brain-barrier is semi-permeable has led to research on exercise as a palliative for disorders of the brain like anxiety, depression, Alzheimer's and multiple sclerosis. Studies from our lab have indicated that activation of the EPO pathway, one critical to conditioning in athletes, is capable of reversing mitochondrial dysfunction in models of MS (Singhal et al. 2015). Human studies on exercise and MS have shown that in some cases, exercise conditioning can improve mobility and cognitive issues (Briken, et al. 2014), but not much is known regarding mechanisms. Because there are multiple types of MS that vary in their severity and progression, it is thought that differential expression of inflammatory molecules at the sites of focal lesioning may determine severity of the disease (Leray et al. 2013). However, it

is clear that MS is driven not only by the loss of conduction in axons resulting from the lesions, but also changes in inflammatory pathways and brain cell metabolism. Our studies on post-mortem tissue from MS brains have shown that nuclear localization of Hbb changes in the course of the disease, and decreases are associated with increased localization to mitochondria and metabolic dysfunction. Mitochondrial respiratory complex loss, fission and decreased oxidative phosphorylation are hallmarks of many neurodegenerative diseases (Compagnoni, et al. 2020). There is a shift from oxidative phosphorylation to increased reliance on glycolytic production of ATP, similar to the Warburg effect in cancer cells. Understanding how the Warburg effect relates to inflammation, glucose metabolism and energy production in the cell is essential to understanding why inflammation impairs the metabolic efficiency of neurons and oligodendrocytes in neurodegenerative disease.

The Warburg effect and neurodegeneration

When ROS levels are sustained at a high level, cells may change their glucose metabolism to a process that resembles anaerobic respiration. This is known as the “Warburg effect”, which was discovered in 1924 by Otto Warburg who observed that despite having a 20% lower respiration rate, cancerous cells exhibit a ten-fold higher glucose uptake than normal cell types (Burns & Manda 2017). During the Warburg effect, cells repeatedly utilize glycolysis rather than aerobic respiration, which poses issues because aerobic respiration has a much greater ATP yield than the lower efficiency anaerobic processes. Raised ROS are known to induce this metabolic shift through activation of HIF-1α, augmentation of sirtuin genes and the NfKb pathway. The precise mechanisms of the switch are elusive in most neurological conditions, but mutations and aberrant activity of the aforementioned players are common in diseases such as Alzheimer’s

and MS. Hypoxia inducible factor 1 alpha (HIF-1α) and the erythropoietin receptor regulate endogenous expression of the hemoglobins within the brain. Low oxygen availability in the short-term creates a need for more efficient oxygen delivery, while impaired mitochondrial activity or a prolonged lack of oxygen may lead to downregulation of expression in order to conserve energy, possibly through the reduced synthesis of heme that occurs when respiration is inhibited. In response to this increased demand for oxygen, the hypoxia inducible factors activate EPO signaling. EPO-associated pathways are therefore major players in metabolic efficiency and cell redox states. The EPO receptor pathway itself is regulated primarily through HIF-2α -dependent mechanisms that control expression of the receptor.

The overarching benefits of exercise for brain health

Exercise has been touted as a potential benefit to the brain for the past few decades, but most research has focused on narrow areas of improvement. Research on memory, depression and cognition has given us BDNF as a major player in the improvement of certain cognitive parameters. BDNF is undoubtedly important for dendritic spine formation and improved working memory. However, there is still scanty evidence regarding the inflammatory milieu and epigenetic changes that may occur within the brain after the addition of physical activity. What we do know is that overall, exercise reduces pro-inflammatory signaling pathways and improves the metabolic health of neurons and other cell types within the brain. Our laboratory has focused on EPO and associated pathways for the improvement of metabolism in neurons and oligodendrocytes (Fig. 25). We have seen increased myelination and reversal of mitochondrial complex loss in the cuprizone model of MS with EPO treatment. Other studies

have shown that high doses of recombinant EPO can prevent grey matter loss and enhance cognition in some neuropsychiatric conditions independently of hematocrit levels (Wustenberg, et al. 2011). In mice, EPO treatment increases adult neurogenesis in the hippocampus (Wakhloo, D. et al. 2020). This suggests a function of EPO in the brain that is independent of hemoglobin as an oxygen carrier. Exercise is an appealing treatment for stimulation of the EPO pathway because it is non-invasive and encourages natural production of the cytokine. We saw upregulation of EPOR and increased Hbb after seven weeks of running in our study, consistent with our expectation that exercise would lead to activation of the EPO pathways. Additionally, the expression of DRP1, the mitochondrial fission protein that can lead to cellular distress in high levels, had a tendency to decrease with exercise. We also know that large scale changes occur in the brain with physical activity. Studies that have been done on the role of oligodendrocytes in motor skill learning have suggested that physical activity leads to firing patterns in the brain that encourage increased myelination of the most relevant pathways (Mackenzie, I.A. et al. 2014).

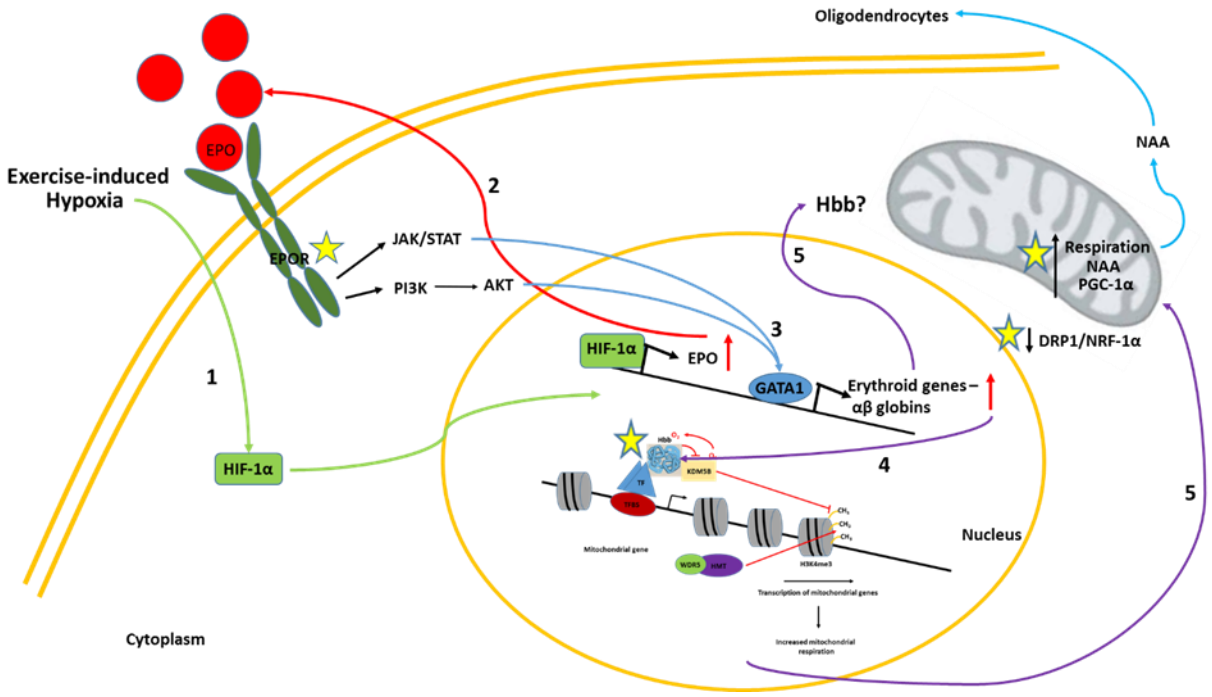


Figure 25. Comprehensive working model for impact of exercise on Hbb expression and mitochondrial function in neurons. Image illustrates the suggested cellular mechanisms and effects of exercise Hbb expression and mitochondrial function in neurons. 1) Exercise serves as a functional challenge to (both physically and perhaps cognitively) induce physiologic hypoxia of involved areas of the brain stimulating HIF 1α. 2) Hypoxia via HIF-1α in turn has been shown to induce EPO production/release in neurons. 3) EPO binds in an autocrine/paracrine fashion to the EPOR whose activation has been linked to increases in transcription of erythroid genes, particularly Hbb. 4) Hbb production has been linked to increases in transcription of genes (see figure 5) and (5) that support increased mitochondrial respiration as evidenced by increased NAA production and oxygen consumption rate. Increased NAA may also provide additional signaling to oligodendrocytes to support myelination and oligodendrocyte energetics. Yellow stars represent steps in the model demonstrated in the work presented here.

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