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THE EFFECTS OF EXERCISE PRECONDITIONING ON FOCAL ISCHEMIC STROKE

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THE EFFECTS OF EXERCISE PRECONDITIONING ON
FOCAL ISCHEMIC STROKE

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science
in the College of Medicine
at the University of Kentucky

By

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2017

ABSTRACT OF THESIS

THE EFFECTS OF EXERCISE PRECONDITIONING ON FOCAL ISCHEMIC STROKE

Cleaved fragments of the extracellular matrix protein perlecan have been shown to promote neuroprotection and repair after ischemic stroke. The cysteine proteases cathepsin B and L as well as the metalloprotease bone morphogenic protein 1 (BMP-1) are capable of releasing the biologically active C-terminal laminin-like globular domain (LG3) of perlecan. Exercise, a known method of reducing stroke risk and severity, has been shown to increase the expression of some proteases associated with perlecan processing. Using a transient distal middle cerebral artery occlusion (MCAo) model for focal ischemic stroke we show that while 7 days of running only slightly decreased infarct volume, *BMP1* and perlecan (*HSPG2*) RNA expression in skeletal muscle was significantly increased in 3-month-old male wild type C57/BL6 mice. Moreover, elevated levels of *BMP1* RNA were still detectable after 3 days of detraining, suggesting a prolonged effect of exercise on *BMP1* expression. Levels of LG3 in the blood were below the limit of detection in the current study, however it is likely that a more sensitive method would enable analysis of serum. These preliminary findings suggest that LG3 could be a molecular mediator of neuroprotection afforded by exercise though further studies are required.

KEYWORDS: Ischemic Stroke, Exercise Preconditioning, Neurovascular Unit,
            Perlecan Laminin-like Globular Domain 3, Skeletal Muscle

Gillian Grohs
July 10th 2017

THE EFFECTS OF EXERCISE PRECONDITIONING ON
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CHAPTER 1: INTRODUCTION

Stroke Epidemiology and Etiology

While the prevalence of stroke has somewhat plateaued in the last 20 years, in part due to widespread statin and anti-platelet use, stroke remains a leading cause of death and disability in the US. Stroke is the second leading cause of death worldwide, with around 6.5 million reported deaths from stroke annually (Benjamin et al., 2017; Kochanek, Murphy, Xu, & Arias, 2014; Ovbiagele & Nguyen-Huynh, 2011). Stroke mortality is an issue in Kentucky in particular, as the state is one of 11 within the "stroke belt" as designated by the National Heart, Lung and Blood Institute. Stroke death rates are 10% higher in the stroke belt than the national average and numerous initiatives have been funded in an effort to increase education and reduce the risk of stroke in these regions (NHLBI, 1996; Thom et al., 2006). A stroke, by definition, is a loss of blood flow in the brain that can cause a localized area of tissue death known as an infarct. Infarcts generally occur when cerebral blood flow (CBF) is less than 10 mL/g of tissue per minute (Latchaw et al., 2003). Consistent blood flow is fundamental in delivering oxygen and nutrients to tissue along with providing an avenue for waste removal; absolutely essential processes in tissues with high metabolic activity such as the brain. Loss of regular circulation can occur due to the physical occlusion of a vessel to cause an ischemic stroke or vessel rupture in a hemorrhagic stroke. Occlusions frequently occur in major intracranial arteries such as the middle cerebral artery (MCA), internal carotid artery (ICA), basilar artery and anterior cerebral artery (ACA). Emergent large vessel occlusion (ELVO) strokes are those in the ICA and proximal segments of the MCA and are associated with high mortality and long-term disability (Jayaraman et al., 2015; Teleb, Ver Hage, Carter, Jayaraman, & McTaggart, 2016). A majority of stroke research, including this thesis, focuses on ischemic ELVO stroke, which make up 87% of stroke cases (Ovbiagele & Nguyen-Huynh, 2011).

Pathophysiology of Ischemia/Reperfusion Injury

In the core of the ischemic lesion, loss of ionic homeostasis leads to metabolic failure that results in rapid tissue death from excitotoxicity, membrane integrity loss and various cellular death mechanisms. Collateral vessels are able to maintain marginal blood flow to the peri-infarcted region, preserving some cellular integrity. This vulnerable tissue surrounding the ischemic core, known as the penumbra, is considerably challenged metabolically and is sensitive to biochemical signals coming from the surrounding tissue and vasculature as well as the primary infarct itself. ATP depletion and ionic homeostasis are drastically compromised with the loss of regular circulation. Dysregulation of the sodium potassium exchanger, in addition to the buildup of CO_2 and lactate from anaerobic respiration, lowers the pH of the tissue and increases the calcium load of cells and mitochondria (Kalogeris, Baines, Krenz, & Korthuis, 2012; Sanada, Komuro, & Kitakaze, 2011). Along with disruption of membrane potential homeostasis, ionic imbalance can drastically alter neurotransmitter release and reuptake, especially in the case of the excitatory neurotransmitter glutamate. Elevated extracellular glutamate levels can cause toxic overstimulation of surrounding cells, which are already depleted of ATP and overloaded with calcium, in a process known as excitotoxicity (Dugan, 1999; Xing, Arai, Lo, & Hommel, 2012). These biological processes can lead to cellular swelling and rupture and can elicit widespread necrosis, but also apoptosis and autophagy; mechanisms that are all present in the ischemic core (Deng, He, Yang, & Zhang, 2016; Xu & Zhang, 2011). The complexity of ischemic injury is further complicated when incorporating the variable of reperfusion of blood in cases where the occlusion is cleared. While the restoration of blood flow provides much needed oxygen and glucose and can reestablish ionic homeostasis, pH levels and eliminate waste and debris; reperfusion can further exacerbate tissue damage as the sudden changes can generate reactive oxygen species (ROS) and proinflammatory signals to prompt additional tissue death

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(Kalogeris et al., 2012; Xing et al., 2012). Not only can reperfusion specifically trigger apoptotic pathways in neurons and resident glial cells; changes in the vasculature itself can exacerbate stress and damage (Manabat et al., 2003; H. Morrison, McKee, & Ritter, 2011; H. W. Morrison & Filosa, 2013). Damage from both the initial ischemia and subsequent reperfusion cause the cells in the penumbra to die slowly over several days as the infarct matures, inevitably resulting in expansion of the ischemic lesion. Many of these more chronic injury mechanisms are mediated through alterations in the vasculature and there is significant research that suggests minimizing these changes significantly reduces injury and disability from ischemic stroke (Lo, Dalkara, & Moskowitz, 2003; Mehta & Vemuganti, 2014).

The Neurovascular Unit

Though vasculature was historically thought of as a straightforward and relatively inert tissue, extensive research has shown that the neurovascular unit (NVU) produces intricate, specific signals to and from surrounding tissue and the whole body. The neurovascular unit is comprised in part of endothelial cells, pericytes, closely associated glial cells, neurons and the surrounding extracellular matrix (ECM) (Lakhan, Kirchgessner, Tepper, & Leonard, 2013; Muoio, Persson, & Sendeski, 2014). The endothelium makes up the innermost layer of blood vessels. Endothelial cells in the brain are exceptionally polarized, have increased numbers of mitochondria, are thinner, and have fewer fenestrations and endocytic vesicles than those in the microvasculature of other parts of the body (Coomber & Stewart, 1986; Roggendorf & Cervós-Navarro, 1977). All of these properties indicate that brain endothelia actively regulate the transcellular transport of various blood constituents into the brain parenchyma. Additionally, specialized proteins are expressed at the junctions of these flat endothelial cells to create a seal that is relatively impermeable to the passive paracellular diffusion of most molecules and many small ions. Transmembrane proteins such as the claudins, occludin and

cadherins are the main components of tight junctions and are tethered to the actin cytoskeleton via cytoplasmic adapter molecules. Tight junction proteins interact strongly through extracellular loops to significantly reduce paracellular transport and also help to maintain the polarity of the apical to basal side of the endothelial cells. Redistribution and reduced expression of tight junction proteins have been demonstrated after stroke and other cerebral inflammatory disorders and are associated with increased edema, leukocyte infiltration, inflammation and overall infarct volume (Jiao, Wang, Liu, Wang, & Xue, 2011; Neuwelt et al., 2011; Stamatovic, Keep, Wang, Jankovic, & Andjelkovic, 2009; Wong et al., 2013; Yang & Rosenberg, 2011). A layer of basal lamina ECM surrounds the endothelial cells to provide structure and support to the neurovascular unit. The basal lamina is made up primarily of laminin, fibronectin, collagen and heparin sulfate proteoglycans. Degradation of the basal lamina and tight junctions by proteases such as the matrix metalloproteases (MMPs) are associated with edema and leukocyte extravasation and can also prompt anoikis (Abbott, Ronnback, & Hansson, 2006; Chen, Ohashi, Li, Eckman, & Nguyen, 2009; Lo et al., 2003). The complexity of the neurovascular unit is markedly increased by additional interactions from cells such as pericytes, astrocytes, microglia and neurons themselves. These cells can influence the expression of tight junctions and ECM proteins, influence transcellular transport across the endothelium, release bioactive factors and effect vasodilation (Abbott et al., 2006; Ben-Zvi et al., 2014; Cheng et al., 2006). All together, these cells and proteins maintain a stable environment for the surrounding brain tissue by creating a blood brain barrier (BBB) that restricts the passage of many circulating elements into the brain parenchyma. All of the aforementioned properties influence permeability of the BBB after ischemia. The BBB does not follow a simple open or closed configuration, as it is constantly responding to signals. The dynamic responses of the NVU are evident as the BBB shows a biphasic opening pattern after stroke. First, a transient opening

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occurs several hours after reperfusion. After an interval of 24 to 48 hours, a second, more sustained increase in permeability, associated with increased vessel damage, takes place (Huang, Xue, Preston, Karbalai, & Buchan, 1999; Knowland et al., 2014; Zlokovic, 2008). Overall, increased permeability of the BBB after stroke is associated with extended tissue damage due to edema and inflammation (Baeten & Akassoglou, 2011; Cuomo et al., 2015). Furthermore, after an ischemic event, upregulation of adhesion molecules on endothelial cells facilitate the infiltration of peripheral leukocytes that can go on to trigger a cascade of inflammatory signaling and damage within the brain (Henninger et al., 1997; Stanimirovic, Wong, Shapiro, & Durkin, 1997).

Contributions of Perlecan to Stroke Neuroprotection

As previously mentioned, activation and release of numerous proteases capable of degrading the ECM can not only break down the BBB, but can also release biologically active fragments from ECM components. A segment of one such ECM protein, perlecan, has demonstrated profound neuroprotective effects when administered after transient ischemia in rodent models of stroke (Bix, Gowing, & Clarkson, 2013; Clarke et al., 2012; Lee et al., 2011). Perlecan is a heparan sulfate proteoglycan that is expressed ubiquitously in basement membranes throughout the body. Proteolytic processing by proteases such as stromelysin and plasmin have been shown to release the C-terminal domain of perlecan: domain V (DV) (Fukuda et al., 2004; Whitelock, Murdoch, Iozzo, & Underwood, 1996). DV can be further subdivided into three laminin-like globular domains, where the terminal domain, LG3, shown to be cleaved and released from DV by bone morphogenic protein-1 (BMP-1), cathepsin B (CTSB) and cathepsin L (CTSL), possesses the highest level of biological activity (Cailhier et al., 2008; Gonzalez et al., 2005; Gu et al., 2015; Saini & Bix, 2012). When administered after experimental stroke, DV has been shown to localize specifically to ischemic regions and significantly reduce infarct volume,

reduce motor and cognitive deficits, increase angiogenesis and reduce inflammation (Bix et al., 2013; Clarke et al., 2012; Lee et al., 2011). These actions have been shown to be accomplished, in part, by interaction with the $\alpha_5\beta_1$ integrin where activation of ERK-signaling pathways can lead to the release of vascular endothelial growth factor (VEGF), a known mediator of neurorepair (Clarke et al., 2012; Zoeller, Whitelock, & Iozzo, 2009). DV has also been shown to act directly on astrocytes via the $\alpha_2\beta_1$ integrin as well as $\alpha_5\beta_1$ to reduce astrogliosis and induce nerve growth factor secretion (Al-Ahmad, Lee, Saini, & Bix, 2011). Recently, running has been shown to increase the expression and release of the LG3 cleaving CTSB into circulation in humans, rodents and non-human primates. Furthermore, the levels of CTSB in plasma correlated with increased memory function in humans (Moon et al., 2016). There is also evidence of increases in CTSL and BMP-1 expression from exercise (Iijima et al., 2016; Schwalm et al., 2015). As proteases, it is likely that these proteins are acting on other factors to release bioactive fragments. Whether LG3 is one of the effectors released in association with exercise is unknown.

Exercise Preconditioning

Risk factors for stroke include age, sex, ethnicity, smoking, hypertension, physical inactivity and diabetes among others (Goldstein et al., 2006). While some risk factors are non-modifiable, several factors are associated with poor cardiovascular health that in many cases can be attributed to low levels of physical activity. The neuroprotective effects of exercise preconditioning have been studied considerably. It has been shown that patients that exercised in the 2 weeks prior to an ischemic event show decreased stroke risk and severity (Deplanque, Masse, Libersa, Leys, & Bordet, 2012; Sacco et al., 1998). These patients showed reduced neurodeficit upon hospital admittance and increased independence one week after stroke. In addition, those that exercised had lower body mass indices and were less likely to have various

modifiable stroke risk factors such as diabetes, hypertension and heart disease (Deplanque et al., 2012; Stroud et al., 2009). Furthermore, reduced infarct volumes, memory and motor deficits, apoptosis, and inflammation have all been demonstrated in animal models of stroke (Curry et al., 2010; Y. H. Ding et al., 2005; Hayes et al., 2008; Liebelt et al., 2010; Shamsaei, Khaksari, Erfani, Rajabi, & Aboutaleb, 2015). Exercise has been shown to increase neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) (Y. Ding et al., 2004; Otsuka et al., 2016), decrease BBB leakiness (Wolff, Davidson, Wrobel, & Toborek, 2015; Yuling Zhang et al., 2013), reduce neuroinflammation (Chio et al., 2017; B. C. Mota et al., 2012; Zhao, Sabirzhanov, Wu, Faden, & Stoica, 2015), attenuate oxidative stress (Endres et al., 2003; Feng et al., 2014), and to reduce resident microglia activation (Kohman, Bhattacharya, Wojcik, & Rhodes, 2013; Zhao et al., 2015). While the association of exercise with improved cardiovascular health, therein reduced stroke risk, is generally accepted, the molecular mechanisms that underlie this neuroprotective effect are multitudinous. Numerous studies have identified various myokines; small peptides released from skeletal muscle into circulation in response to muscle contractions associated with exercise (Endres et al., 2003; Moon et al., 2016; Schnyder & Handschin, 2015). Identification and characterization of a secreted component that influences a range of neuroprotective mechanisms would be of great interest for adaption to therapeutic use.

Study Summary

We hypothesize that exercise will increase the production and release of LG3 from skeletal muscle into systemic circulation through the increased expression of proteases associated with LG3 cleavage. We expect circulating LG3 to localize to the infarcted regions where the levels of which are also expected to correlate with reduced stroke volume, BBB breakdown and neuroinflammation. Here, changes in RNA and protein expression were evaluated after 7 days of

running wheel exposure in 3-month-old wild type male C57/BL6 mice. Expression of the CTSB and BMP-1 proteases in type II fast twitch gastrocnemius muscle were evaluated in stroked and naïve mice using real-time polymerase chain reaction (PCR) and protein immunobloting. A transient distal middle cerebral artery occlusion model (MCAo) was used to evaluate the effect of running specifically on focal cortical ischemia/reperfusion injury in contrast to a majority of previous studies that have utilized global and central cerebral ischemia models. Ischemic and peri-infarcted cortical regions were evaluated for markers of inflammation and BBB integrity. The aim of this study was to investigate the potential of exercise to induce LG3 release and to correlate these measures with markers of stroke pathology. A graphical representation of the study hypotheses is depicted in Figure 1.1.
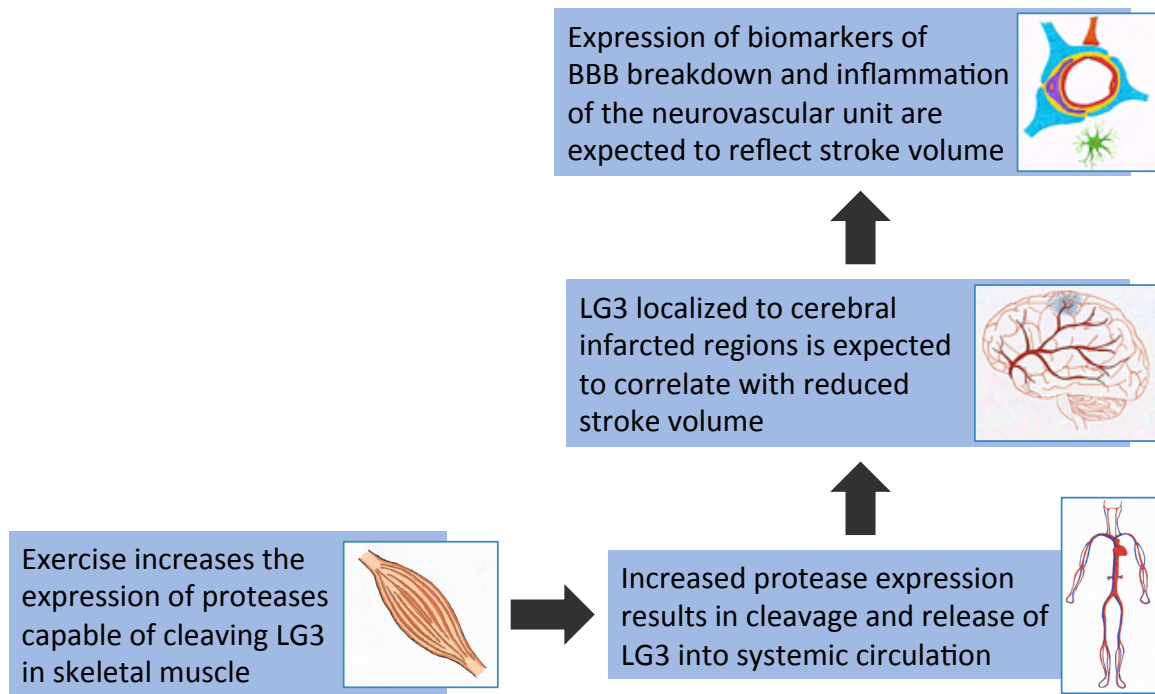
Figure 1.1 Overall study hypotheses.

Exercise, in the form of voluntary running, is expected to increase the expression of perlecan cleaving proteases in skeletal muscles utilized during physical activity. This increase in protease expression is anticipated to result in the generation of the LG3 fragment of perlecan that will be subsequently released into systemic circulation. Localization of LG3 to regions of the brain that have undergone an ischemic insult is predicted to reduce stroke volume by reducing BBB breakdown and neuroinflammation of the NVU.

CHAPTER 2: MATERIALS AND METHODS

Animals and Exercise Paradigm

All experimental procedures were performed in accordance with the University of Kentucky Division of Laboratory Animal Research, Institutional Animal Care and Use Committee guidelines under protocol number 2015-2156. 12-week-old male C57/BL6 mice from Jackson Laboratories were housed individually in mouse single activity wheel chambers (Lafayette Instruments, Lafayette, IN) with disassembled running wheels for 1 week of acclimation prior to any experiments and were provided food and water ab libitum for the duration of study. Animals were separated into 5 groups (Figure 2.1), consisting of 3 naïve groups: 7 days of free access to running wheel (7d RW, n=4); sedentary controls that were housed in identical running wheel cages for 7 days where the running wheel had been removed in order to control for single housing (sedentary, n=4); and 7 days of free running wheel access with wheel removal on day 7 followed by 3 sedentary days in order to reproduce the 3 day sedentary interval experienced by the surgery groups (7d RW + 3d sed.). The two surgery groups included: 7 days of free access to running wheel with wheel removal and MCAo on day 7 followed by a 3 day sedentary period (7d RW + MCAo, n=6), and 7 days of sedentary control with MCAo on day 7 followed by an additional 3 days of sedentary housing (sedentary + MCAo, n=6). As the aim of the current study was to analyze the effects of exercise prior to stroke it was necessary to remove the running wheels after surgery in order to focus on preconditioning rather than introduce the variable of exercise rehabilitation after stroke. Animals with access to a running wheel are considered "exercised" in the current study. Running wheel revolutions were detected and recorded by Lafayette Instruments Activity Wheel Monitor software v11.12. Animals ran voluntarily and predominantly during the dark period of the animal facility light cycle (2100-0700 local time).
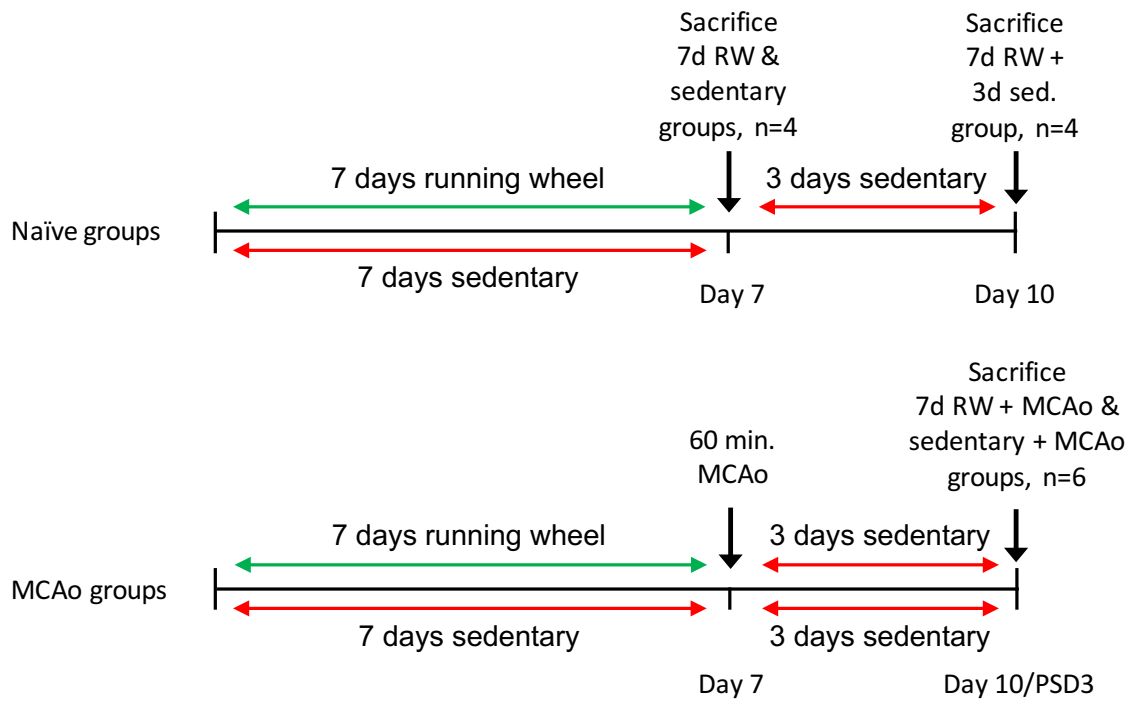
Figure 2.1 Running wheel study design.

Animals were housed in cages where running wheels had been disassembled during sedentary intervals to control for the effects of single housing. Serum was taken via submandibular punch prior to sacrifice. Naïve groups (7d RW, sedentary and 7dRW + 3d sed.) were transcardially perfused with phosphate buffered saline (PBS) prior to tissue harvesting; cervical dislocation without perfusion was performed on MCAo groups (7d RW + MCAo and sedentary + MCAo).

Model of Cerebral Ischemia

Mice from the 7d RW + MCAo and sedentary +MCAo groups were anesthetized with an 87.5 mg/kg ketamine/12.5 mg/kg xylazine cocktail via intraperitoneal injection. Ischemia/reperfusion was induced via the transient tandem ipsilateral common carotid artery (CCA)/MCA model (Waxham, Grotta, Silva, Strong, & Aronowski, 1996) for 60 minutes followed by reperfusion for 3 days to post stroke day 3 (PSD3). MCAo was performed between 5 and 8 hours after the end of the light cycle. Briefly, a small burr hole was drilled over the left MCA and a 0.005-inch diameter stainless-steel wire filament was slid under the MCA and placed over the skull using the sides of the gap created by the burr hole to elevate the MCA and create tension to occlude the artery. A >80% reduction of blood flow was confirmed via laser-Doppler flowmetry using a Laser Doppler Perfusion Monitor (Perimed, Ardmore, PA) placed distally to the filament. The CCA was then isolated and occluded with an aneurysm clip to reduce collateral perfusion of the MCA territory by the ACA. After 60 minutes, both the CCA clip and MCA filament were removed and reestablishment of >75% original blood flow was confirmed using laser-Doppler flowmetry. Infarct volumes were analyzed on PSD3 using 2,3-triphenyltetrazolium chloride (TTC), where tissue with functional mitochondria stains a red color and infarcted tissue with damaged mitochondria remains white. Brains were cut into 2-mm thick coronal sections and stained with TTC for 12-15 minutes at room temperature. Infarct volumes were calculated using three 2mm coronal brain sections by computer-assisted volumetry using the following equation:

$$Infarct\ volume = d \sum_i \left(F_i \left(\frac{C_i}{I_i} \right) \right)$$

Equation 2.1 Infarct volumes were normalized for swelling using the above equation where d is the thickness of the section (2 mm), F_i is the measured area of ischemia, I_i is the total area of the ipsilateral hemisphere, and C_i is the total area of the contralateral hemisphere for slice i.

Tissue Processing

In accordance with the timing depicted in figure 2.1, naïve animals from the 7d RW, sedentary and 7d RW + 3d sed. groups were deeply anesthetized with ketamine/xylazine and transcardially perfused with PBS prior to isolation of gastrocnemius muscles. In order to utilize TTC staining for robust infarct visualization, perfusion was not performed on MCAo groups; therefore these animals were sacrificed via cervical dislocation. After TTC staining, the infarcted cortex along with a modest amount of peri-infarcted tissue was dissected from each brain section. All brain tissue and gastrocnemius muscles were flash frozen in liquid nitrogen. Blood was collected prior to sacrifice via a submandibular punch into serum separator tubes, allowed to clot and centrifuged at 2000 rcf for 15 minutes at 25°C. Serum and all tissue samples were stored at -20°C.

Reverse and Real-time Transcription Polymerase Chain Reaction (PCR)

Total RNA was isolated from tissue using a rotor tissue homogenizer and Trizol™ Reagent (ThermoFisher, Carlsbad, CA) with a 5:1 ratio of chloroform. RNA was then purified using a spin column-based PureLink™ RNA Mini kit (Life Technologies, Carlsbad, CA). RNA concentration was measured using a NanoDrop™ spectrophotometer. cDNA was produced using a High-Capacity cDNA Reverse Transcription kit (Life Technologies, Carlsbad, CA) according to manufacturer's instructions. Real-time PCR was performed using FAM™-MGB reporter-quencher probes with TaqMan™ Fast Advance Master mix using 50 ng total RNA per sample. Target amplification was performed using the ViiA™ 7 Real-Time PCR system and associated software v1.2 (Applied Biosystems, Grand Island, NY). Thermal cycling conditions were as follows: 95°C holding stage, 40 cycles of 1 second 95°C denaturing and 60°C annealing/primer extension for 20 seconds. Table 2.1 describes the genes analyzed along with RefSeq and TaqMan identifiers.

Table 2.1 List of genes probed in real-time PCR assays.

GENE OF INTEREST	REFSEQ ID	TAQMAN ID
Cathepsin B (CTSB)	NM_007798.3	Mm01310506_m1
Bone Morphogenic Protein 1 (BMP1)	NM_009755.3	Mm00802220_m1
Perlecan (HSPG2)	NM_008305.3	Mm01181173_g1
Peroxisome Proliferative Activated Receptor Gamma, Co-activator 1 Alpha (PPARGC1A)	NM_008904.2	Mm01208835_m1
Occludin (OCLN)	NM_008756.2	Mm00500912_m1
Claudin-5 (CLDN5)	NM_013805.4	Mm00727012_s1
Matrix Metallopeptidase 9 (MMP9)	NM_013599.3	Mm00442991_m1
Intracellular Adhesion Molecule 2 (ICAM2)	NM_010494.1	Mm00494862_m1
Vascular Cell Adhesion Molecule 1 (VCAM1)	NM_011693.3	Mm01320970_m1
Integrin alpha 5 (ITGA5)	NM_010577.3	Mm00439797_m1

Gene expression normalized to 18S rRNA or RPL38 ribosomal protein. Probe sequences are proprietary information of Thermo Fisher Scientific.

Western Immunoblots

Lysates were prepared from flash frozen tissue in ice-cold RIPA lysis buffer with protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) using a rotor tissue homogenizer. Homogenates were centrifuged at 20000 x g for 15 minutes at 4°C. Pierce™ bicinchoninic acid (BCA) protein assay kit was used to determine protein concentration. 30 μg total protein was mixed with glycerol/SDS protein loading buffer (LI-COR, Lincoln, NE) and denatured with 2-Mercaptoethanol at 95°C for 5 minutes. Samples were loaded into 4-20% Mini-PROTEAN® TGX™ precast gels (Bio-Rad, Hercules, CA), SDS-PAGE was used to separate proteins, and iBlot® transfer system was used to transfer proteins to a nitrocellulose membrane. Membranes were blocked in LI-COR Odyssey® Blocking Buffer (Lincoln, NE) for 1 hour at room temperature on an orbital shaker. Primary antibodies were incubated in blocking buffer with 0.1% Tween overnight at 4°C on a see-saw shaker. Membranes were probed with anti-pan cathepsin antibody (H-1) sc376803, 1:250 dilution (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-LG3 antibody (H-300) sc-25848, 1:250 dilution (Santa Cruz Biotechnology, Santa Cruz, CA). An anti-GAPDH antibody, 1:10000, was used for gastrocnemius protein normalization (GeneTex, Ivine, CA). Near-infrared fluorescent secondary antibodies (LI-COR, Lincoln, NE) were incubated for 1 hour at room temperature in blocking buffer with 0.1% Tween and 0.01% SDS. Visualization using the LI-COR Odyssey® Infrared Imaging System was used for semi-quantitative protein concentration analyses.

Statistical Analysis

Data are presented as mean ± standard deviation unless otherwise noted. Statistical analysis was performed using GraphPad Prism statistical package version 7.0b for Mac OS (GraphPad Prism, La Jolla, CA).

CHAPTER 3: RESULTS

Validation of Running Wheel Group Comparisons

Daily running wheel distances were logged and compared for variation between groups in order to confirm that animals exercised at similar levels (Figure 3.1). Animals that were sacrificed immediately after a 7-day running wheel interval (7d RW group) ran an average of 8263 ± 1198 meters per day and a 7-day cumulative average of 49.7 ± 9.9 km, n=4. Animals that were subjected to MCAo after the 7-day running wheel interval and were then housed in cages without running wheels for 3 days prior to sacrifice (7d RW + MCAo group) ran an average of 7100 ± 749 meters per day and 57.8 ± 7.6 km cumulative, n=6. Animals that were housed in running wheel cages for 7-days prior to removal of wheels for a 3-day sedentary period (7d RW + 3d sed. group) ran an average of 6788 ± 1244 meters per day, 47.5 ± 25.7 km cumulative, n =4. There were no significant differences between the daily averages of the groups, $p=0.54$, or the cumulative distances, $p=0.54$, therefore comparison between groups is acceptable (Ordinary one-way ANOVA). Distance run per day over time did significantly increase as all animals ran an average of 7389 ± 2746 m on day 1 and 8489 ± 2601 m on day 7, $p<0.01$ (Two-way ANOVA).
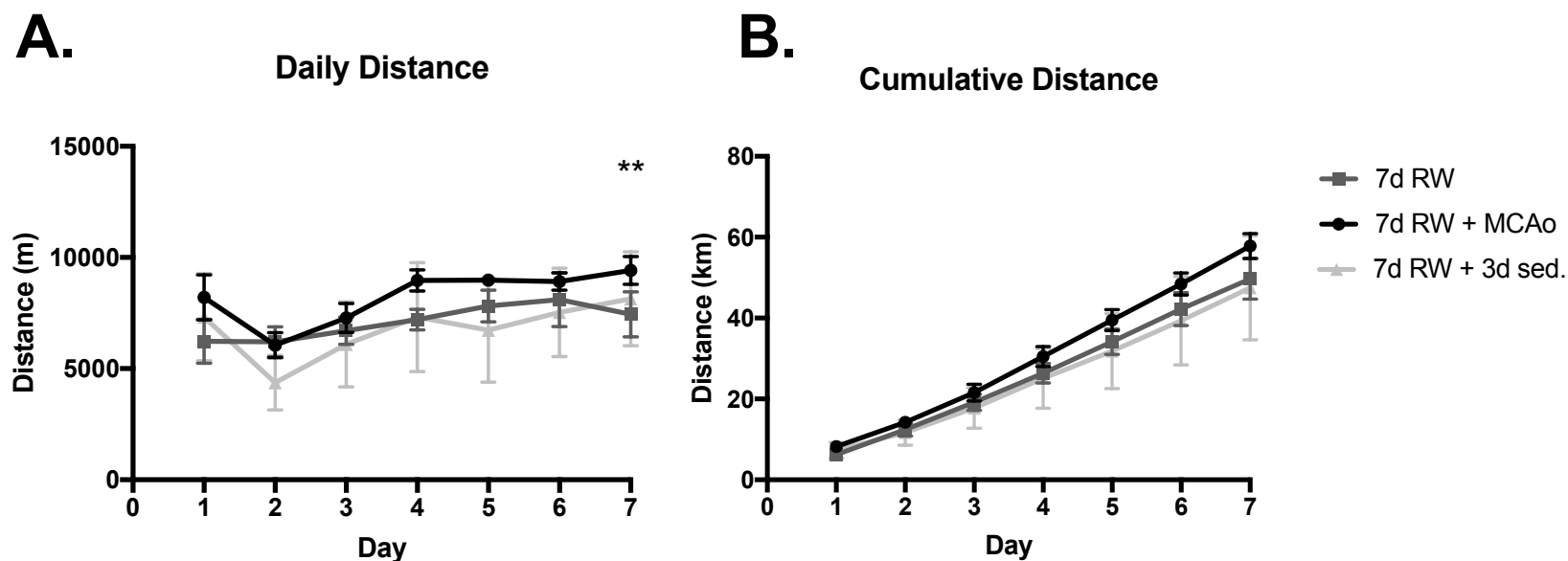
Figure 3.1 There is no significant variation in running wheel distances between groups.

(A) There was no significant difference in the average distance run per day between any of the groups. There was a significant interaction between distance run and time (Two-way ANOVA **p<0.01) where animals ran more on day 7 than they had on day 1. (B) Cumulative running wheel distances were not statistically different between groups. Data points represent average running wheel distance within the group. n=6 for RW + MCAo, n=4 for 7d RW and 7d RW + 3d sed. groups. Data presented as mean ± SEM.

Effect of 7 days of Exercise on Infarct Volume

Infarct volumes from animals in the 7d RW + MCAo and sedentary + MCAo groups were analyzed on PSD3 using TTC staining, where healthy tissue stains red and infarcted tissue remains white (Figure 3.2). One animal in each group displayed aspects of blood within the infarcted region consistent with hemorrhage and were excluded from the study. Animals that had run for 7 days prior to MCAo had slightly smaller infarcts than those in the sedentary control group (29.7 ± 4.6 mm^3 vs. 22.8 ± 6.6 mm^3, p=0.0895, n=5). Though running distances between the groups were not statistically significant (Figure 3.1) there was some variation within the groups. Infarct volumes were therefore compared with running wheel distances to evaluate potential correlations (Figure 3.3). There was no correlation between the cumulative distance run or the average distance run per day to infarct volume (Pearson correlation= -0.048, p=0.9, n=5 for both tests). There was, however, loose correlation between the distance run on the final day of exercise just prior to MCAo and infarct volume though this was not statistically significant (Pearson correlation= -.564, p=0.32, n=5). Animals ran primarily during the dark periods of the animal facility light cycle (2100-0700 local time) and surgeries were performed between 1000 and 1300 local time; therefore animals were occluded within 3 to 6 hours of running. We next evaluated potential changes in protease expression within skeletal muscle to investigate if any changes reflect the data derived from infarct volume.

A.

B. Sedentary + MCAo   7d RW + MCAo

p=0.0985
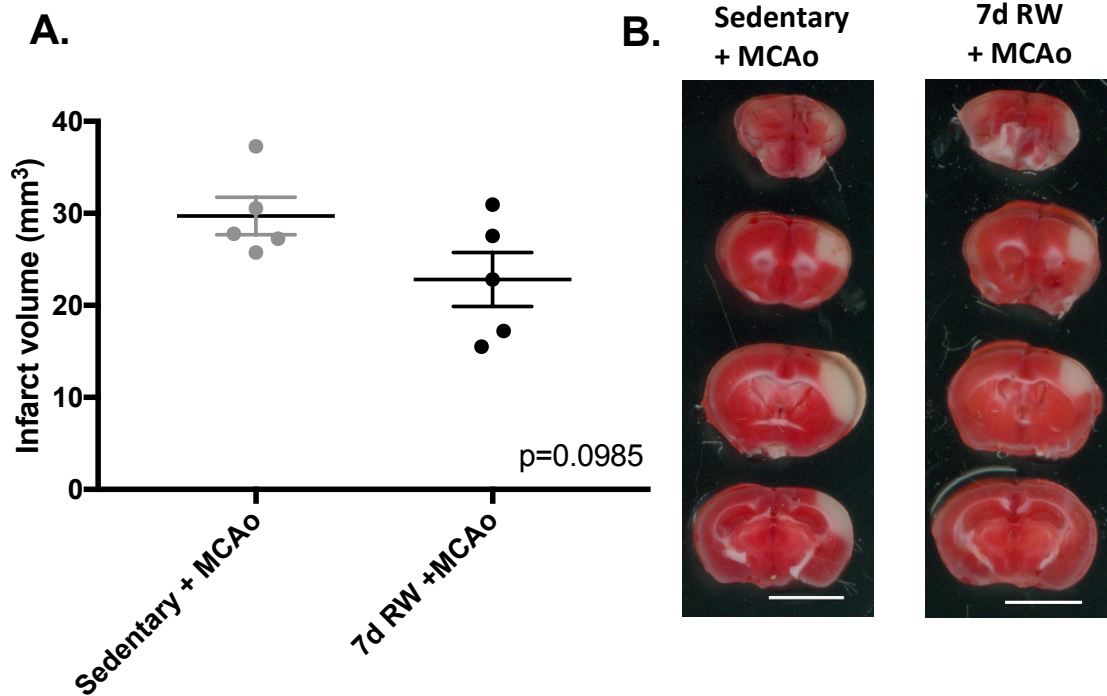
Figure 3.2 Exercised mice have slightly smaller infarct volumes on PSD3.

(A) Animals subjected to MCAo after 7 days of housing in running wheel cages had smaller infarct volumes than sedentary controls on PSD3 (29.7 ± 4.6 mm^3 vs. 22.8 ± 6.6 mm^3) though the difference was not statistically significant (p=0.0985, Student's t-test, n=5). (B) Representative images of coronal brain sections from sedentary and exercised animals used for infarct volume analysis. Tissue stained with TTC to visualize infarcted areas, equation 2.1 was used to calculate infarct volume. White arrows indicate 0.0 bregma estimate, scale bars depict 5 mm.
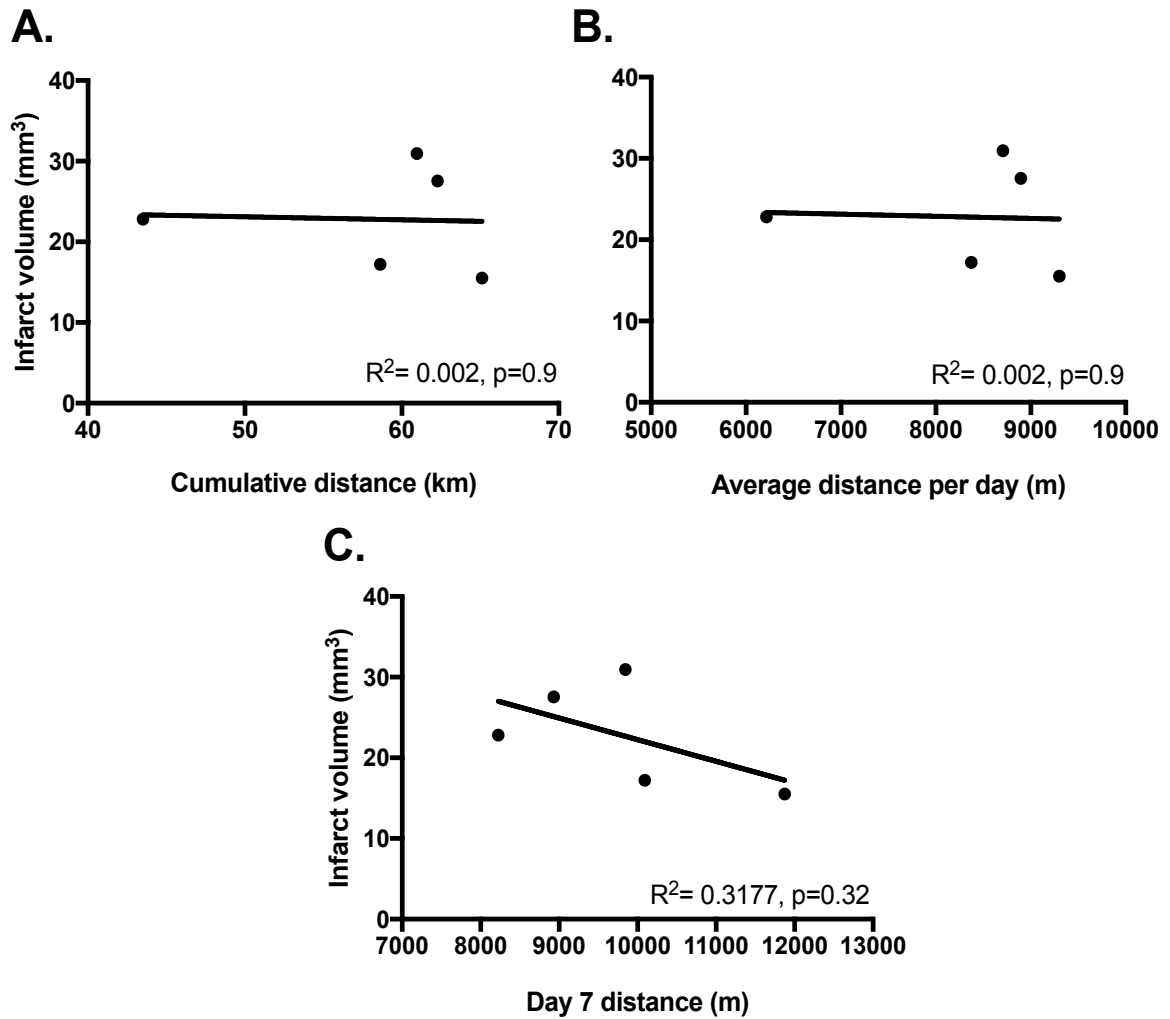
Figure 3.3 Infarct volumes do not correlate significantly with 7 day running wheel distances.
Correlations between (A) cumulative distance, (B) average daily distance and (C) distance on day 7 just prior to MCAo were not statistically significant. Distance run on day 7 showed the highest correlation of the three comparisons (Pearson correlation = -0.048, -0.048 and -0.564 respectively, n=5).

Protease Expression after 7 days of Exercise

Gastrocnemius muscle tissue from all groups was analyzed with real-time PCR to investigate changes in the expression of the CTSB and BMP1 proteases previously shown to be capable of cleaving LG3 from the full-length perlecan molecule. RNA levels of *CTSB* and *BMP1* were compared to levels in sedentary, naïve control mice using *RPL38* as an internal control (Figure 3.4 A &B). *CTSB* levels were not significantly different in any of the groups (One-way ANOVA, p=0.6425, n=4 for naïve groups, n=5 for MCAo groups). Protein expression levels of pro- and mature forms of CTSB in gastrocnemius muscle tissue were analyzed using Western blotting to determine if the RNA levels reflected protein expression (Figure 3.4. C-E). No differences were detected between the naïve sedentary mice or exercised groups (One-way ANOVA, p=0.09, n=4). There was also no difference in CTSB between the MCAo groups (Student's t-test, p=0.48, n=5). *BMP1* RNA levels, however, did show significant differences among groups. *BMP1* levels in gastrocnemius tissue from mice that ran for 7 days was increased 1.4 ± 0.12-fold over naïve sedentary mice (One-way ANOVA with Dunnett's post hoc analysis **p<0.01, n=3). A 1.3 ± 0.15-fold increase over sedentary controls was maintained after a 3-day sedentary period (One-way ANOVA with Dunnett's post hoc test *p<0.05, n=4). *BMP1* was not, however, significantly elevated in either of the MCAo groups. *BMP1* in the sedentary + MCAo was very similar to that detected in the naïve sedentary group. The 7d RW + MCAo group displayed a trend of slightly elevated levels of *BMP1* compared to control. These data indicate that running itself is responsible for the increase in *BMP1* rather than the physiological changes that arise after an experimental stroke injury. We then wanted to determine if exercise was inducing changes in perlecan expression and to also assess whether the 7-day exercise paradigm used in the current study is capable of producing a change in muscle markers of exercise training.

A. *CTSB*

B. *BMP1*

*p>0.05
**p>0.01

C.

Pro-CTSB
CTSB
GAPDH

7d RW   Sedentary   7d RW + 3d sed.

D.

Pro-CTSB
CTSB
GAPDH

7d RW + MCAo   Sedentary + MCAo

E. CTSB protein expression

F. CTSB protein expression
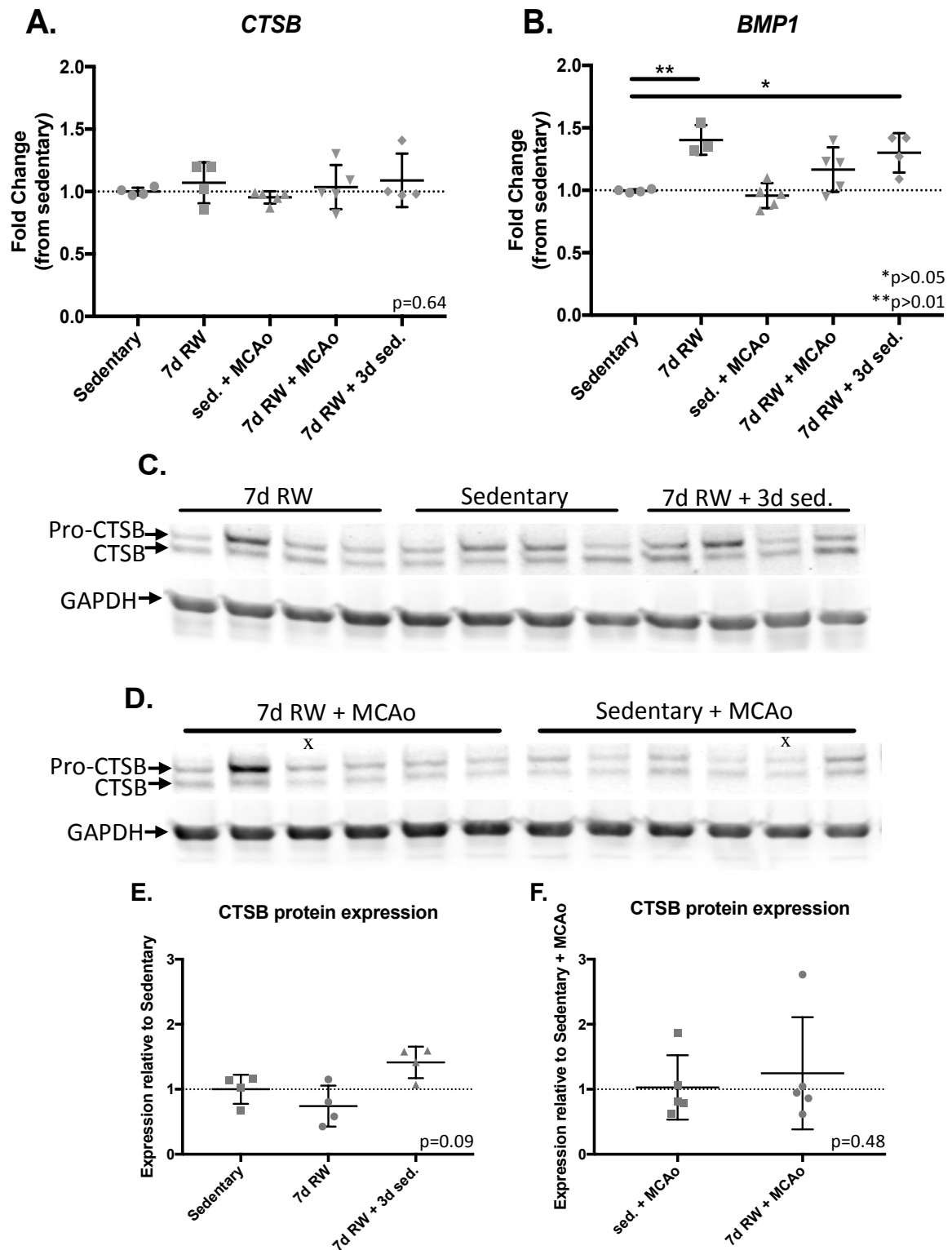
Figure 3.4 7 days of running wheel significantly changes expression of *BMP1* RNA but does not change CTSB RNA or protein levels in gastrocnemius skeletal muscle.

Figure 3.4 cont. (A) Significant differences in the expression of *CTSB* RNA in gastrocnemius muscle were not detected using real-time PCR. (B) *BMP1* RNA was found to be significantly increased in naïve animals that ran for 7 days and was still elevated after a subsequent 3-day sedentary period (One-way ANOVA with Dunnett's post hoc test **p<0.01, *p<0.05, n=3, n=4 respectively). Animals that had undergone MCAo did not exhibit significant differences in *BMP1* from sedentary naïve controls, though levels in the 7d RW + MCAo showed an elevated trend. (C,E) Total protein levels of CTSB were not significantly different between sedentary, 7 day running wheel or 7 day running wheel with 3-day sedentary period. (D,F) There was also no difference in CTSB protein levels between running and sedentary mice that underwent MCAo. (x) indicates animals that were removed from study due to hemorrhage. RNA normalized to *RPL38* internal control, naïve sedentary group used to calculate fold change. Western blots normalized to GAPDH.

Perlecan and PPARGC1A RNA Expression after Exercise

Analyses of total perlecan RNA levels (*HSPG2*) were used to speculate if increases in perlecan expression itself could contribute to any potential increases in LG3 levels. *HSPG2* expression was significantly upregulated after 7 days of exercise in naïve animals (1.24 ± 0.39-fold, One-way ANOVA with Dunnett's post hoc test **$p<0.01$, n=4) (Figure 3.5 A). This increase, however, was lost after 3 days of detraining (both MCAo groups had running wheels removed for 3 days post-surgery prior to tissue harvesting). As a master regulator of mitochondria biogenesis and adaptation of muscle endurance in response to exercise (Handschin & Spiegelman, 2008; Ruas et al., 2012; Russell et al., 2003), *PPARGC1A* expression was used to measure training effect. There was only a slight increase in the 7-day running wheel group compared to naïve sedentary control mice (1.06 ± 0.17-fold), all other groups displayed expression levels slightly lower than control. This lack of *PPARGC1A* upregulation indicates that changes in transcriptional profiles indicative of exercise training were not induced to a significant amount in the gastrocnemius after 7 days of voluntary running. Since the levels of *BMP1* and *HSPG2* were upregulated by this training regime, we next wanted to investigate the potential of LG3 release into serum.
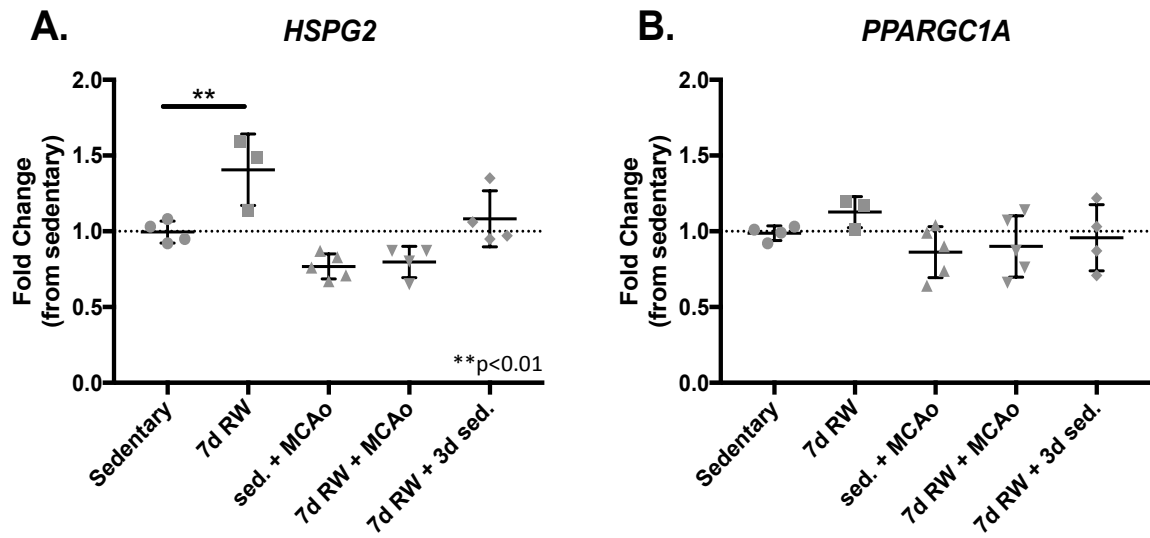
Figure 3.5 *HSPG2* levels are significantly increased after 7 days of running and return to baseline after a 3-day sedentary period though *PPARGC1A* did not change.

HSPG2 was significantly upregulated in the 7-day running wheel group (One-way ANOVA with Dunnett's post hoc test, $**p<0.01$, n=4). Levels returned to baseline in both MCAo groups as well as in naïve mice with running wheels removed for 3 days. *PPARGC1A* levels were not significantly different between any of the sedentary or running wheel groups (n=4 in naïve groups, n=5 in MCAo groups) signifying a lack of training effect.

Serum LG3 Levels

Serum collected at the time of sacrifice was analyzed for the presence of LG3 (Figure 3.6).

Serum samples from all groups were filtered through 50 kDa size exclusion filters to reduce the

interference of IgG light chain (25 kDa) and albumin during electrophoresis and detection.

Albumin has been shown to compose 50% to 60% of serum protein and is not completely

removed by filters to frequently produce a non-specific band of 65 kDa (Peters Jr, 1995). Purified

murine LG3 protein was run alongside serum samples as a positive antibody control for LG3 (~26

kDa). A band corresponding to the molecular weight of LG3 was undetectable in all samples

(Figure 3.6 A & C). Various concentrations of purified murine LG3 were mixed with serum and

filtered as additional positive controls on separate Western blots where a light band

corresponding to the molecular weight of LG3 was detected from 10 μL of 0.6 μg/mL LG3

(estimated 6 ng total LG3 present on membrane) (◊ Figure 3.6 B). LG3 was undetectable when

analyzed at a concentration of 0.1 μg/mL (1 ng total LG3). Therefore it is possible that LG3 levels

are less than 0.9 μg/mL and/or more than 15 μL total serum is required for analysis. There was

also no evidence of DV (~85 kDa), though it is expected that a majority of DV would be removed

during size exclusion filtering.
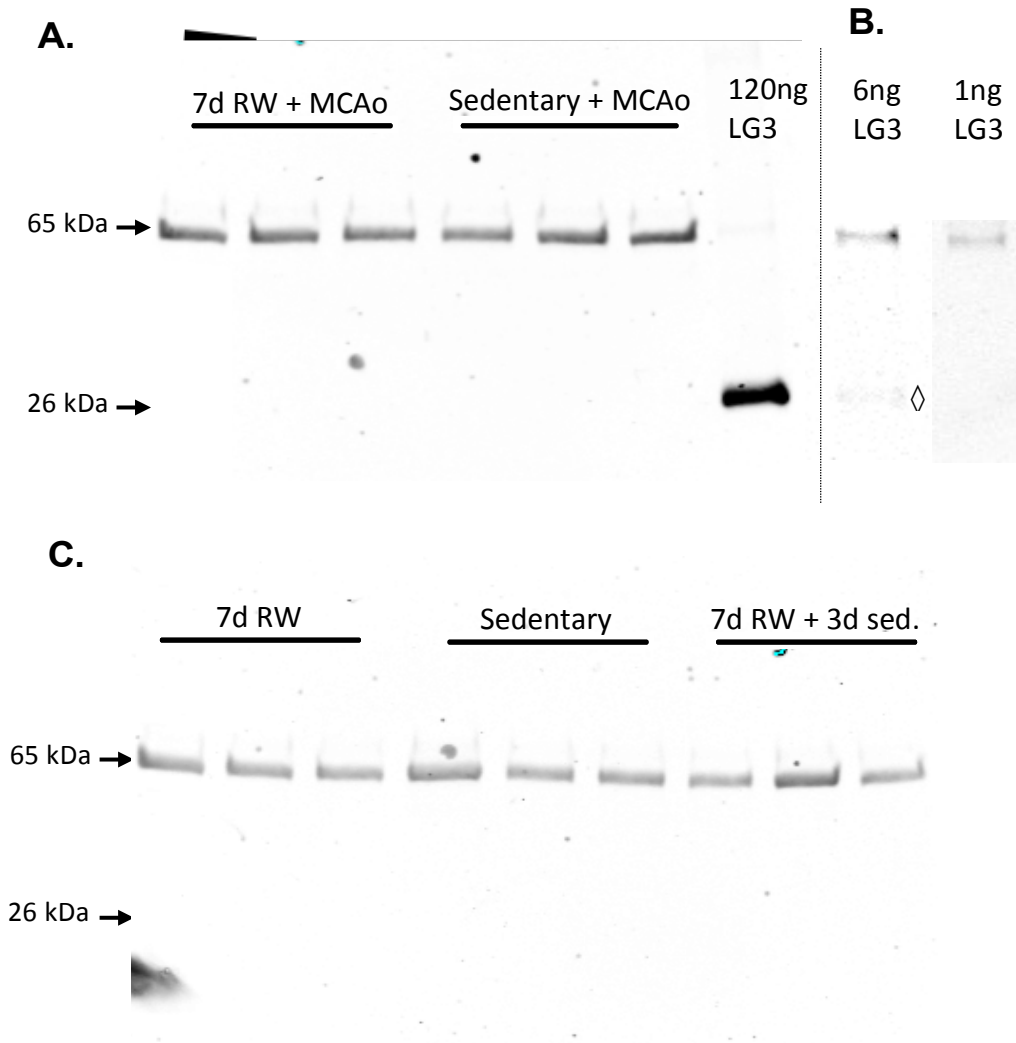
Figure 3.6 Serum levels of LG3 are too low for detection via Western blot

15 µL of total serum was analyzed per animal for the presence of LG3. (A & C) A 65 kDa band corresponding to the molecular weight of albumin was detected in all samples, however, there was no band detected at the molecular weight corresponding to LG3 in any study samples (26 kDa). 120 ng of purified recombinant murine LG3 was also analyzed for positive antibody control (A). (B) Immunoblot detection limits were evaluated by adding recombinant murine LG3 to normal serum prior to 50 kDa size exclusion filtration. A faint band corresponding to the molecular weight of LG3 was detectable in 10 µL total serum with 0.6 µg/mL LG3 added for a total mass of 6 ng (◊), however, this band was undetectable at a concentration of 0.1 µg/mL, total mass of 1 ng.

Cortical RNA Expression of Tight Junctions and Inflammatory Markers After MCAo

Following TTC staining for infarct analysis, ischemic areas along with a portion of the cortical penumbra region were analyzed for changes in gene expression between the exercised and sedentary mice after MCAo (Figure 3.7). All samples were normalized to an *18S* internal control. There was no difference in occludin and claudin-5 RNA expression between exercised and sedentary control animals, suggesting a similar status of BBB integrity. Markers of inflammation: *MMP9, ICAM2,* and *VCAM1* displayed increased variance within the groups compared to occludin and claudin-5 but still did not demonstrate statistical differences in the infarcted regions of exercised and sedentary mice. Additionally, expression of the prototypical LG3 receptor, $\alpha 5$ integrin, was also unchanged. Together these data suggest that the slight reduction observed in infarct volume cannot be definitively attributed to an exercise induced reduction in BBB breakdown or neuroinflammation. The lack of LG3 receptor upregulation could be reflective of the absence of neuroprotection that is expected to be produced from exercise through maintenance of the NVU after stroke.
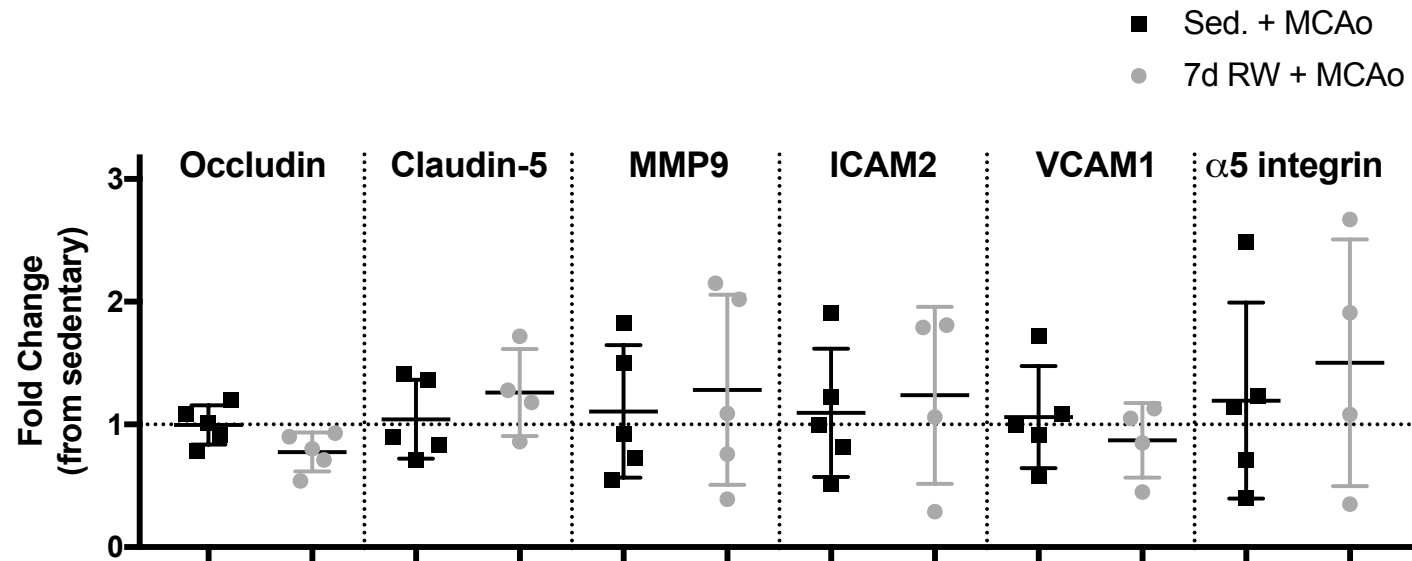
Figure 3.7 There is no difference in the gene expression of tight junctions or inflammatory markers after MCAo in the cortex of 7d RW and sedentary mice.

RNA from ischemic and peri-infarcted cortical regions was analyzed for changes in expression between exercised and sedentary animals. No significant changes were detected in markers for BBB integrity or neuroinflammation.

CHAPTER 4: DISCUSSION

To analyze the effect of 7 days of exercise on ischemic infarct volume we used the distal transient MCAo model of experimental stroke. Results from this study showed that 3-month-old, wild type male C57/BL6 mice caged in running wheel cages for 7 days had slightly smaller ischemic infarcts after 60 minutes of distal MCAo, a model that has not been used previously with exercise preconditioning. The size of the infarct did not correlate with cumulative distance or daily average distance of running. There was, however, a correlative trend between infarct size and distance run during the night prior to surgery.

Many previous studies have used longer periods of 2 to 4 weeks of running and many have used rats rather than mice (Y. H. Ding et al., 2005; Liebelt et al., 2010; Shamsaei et al., 2015; Q. Zhang, Zhang, Yang, Wan, & Jia, 2014). A majority of these studies have also used an intraluminal filament model to produce occlusions at the bifurcation of the middle cerebral artery and anterior cerebral artery from the internal carotid artery rather than the distal model used in the current study. The MCAO designation is used to differentiate the medial MCA occlusion versus the distal MCAo model. In the MCAo model, the MCA is occluded at the M4 segment distal to the M2, M3 and lenticulostriate vessels that perfuse major portions of the basal ganglia and insular cortex (Aronowski et al., 1994). Occluding the MCA at a more medial location that encompasses all branches of the MCA in the MCAO model produces larger infarcts that involve not only frontal and parietal cortical regions but also subcortical regions such as the hippocampus, striatum, thalamus and midbrain (Macrae, 2011). Since the infarct volumes are smaller in the MCAo model, affecting only the cortex, the effect of any intervention needs to be quite robust for detection in a study with a small number of animals. Power analysis of the current study indicates a group size of 15 is estimated to detect a significant difference using α = 0.05 and d = 0.80 for 20% effect size (Snedecor & Cochran, 2017) if the populations are indeed

different. Increasing subject numbers could reveal a significant effect of 7 days of exercise on infarct size, however, in many previous exercise studies animal numbers range from 5 to 10 per group with a difference of 20-30% in infarct volume. These results have been demonstrated in both exercised rats and mice, though this was using the MCAO model (Y. H. Ding et al., 2005; Endres et al., 2003; Hayes et al., 2008; Liebelt et al., 2010; Q. Zhang et al., 2014). It is important to note, however, that most human strokes are relatively small in size, ranging from 4.5-14% of hemispheric volume and so the MCAO model may not reflect the typical clinical condition (Carmichael, 2005). In the current study, the pooled average infarct volume was 17.1 ± 1.8% of the ipsilateral hemisphere, compared to an range of 21% to 45% in many studies using the MCAO model (Carmichael, 2005). In human studies, infarcts larger than 39% of the hemisphere are considered malignant infarcts and are associated with mortality rates up to 80%. This type of stroke makes up 10% of clinical stroke cases and there are few effective treatments for these patients (Hacke et al., 1996). Therein, the smaller cortical infarct produced by the MCAo is likely the most physiologically relevant model.

Given these data, increasing the number of animals in the current study could enable detection of a statistical difference from 7 days of running on infarct volume, however, the robustness of the molecular changes associated with this difference may not be physiologically relevant. While there were no differences in inflammatory marker gene expression in the current study, others have shown a significant reduction after longer intervals of exercise preconditioning. Y. H. Ding et al. (2005) showed that rats that underwent 3 weeks of treadmill training displayed reduced ICAM-1 mRNA expression in the frontoparietal cortex that was associated with reduced peripheral leukocyte infiltration after MCAO compared to sedentary controls. This reduced neuroinflammation also corresponded with a 40% reduction in infarct volume, supporting the hypothesis that exercise preconditioning may reduce damage by

mitigating the surge in neuroinflammation after stroke. There is also evidence for the neuroprotective effect of exercise against other forms of brain injury such as traumatic brain injury (TBI). Four weeks of treadmill training was shown to both reduce levels of the pro-inflammatory cytokine interleukin (IL) 1β (IL-1β) as well as increase anti-inflammatory IL-10 after TBI (B. Mota et al., 2012). Aerobic training in humans has been shown to reduce levels of MMP9 as well as MMP2 (Nascimento Dda et al., 2015), which is considered to correlate with increased BBB integrity. Not only is exercise protective in the healthy animals used in most studies, the effects of exercise are also beneficial in animals with co-morbidities. Reduction of BBB integrity has been demonstrated in the streptozotocin induced diabetic mouse model and correlated with decreased occludin expression (Hawkins, Lundeen, Norwood, Brooks, & Egleton, 2007). In this same diabetic model, 4 weeks of treadmill training maintained claudin-5 levels that were reduced in sedentary streptozotocin mice (de Senna et al., 2015). These findings further support the substantial role of exercise in preserving the BBB via reduction of ECM and tight junction degradation; that along with the expression of proinflammatory cytokines, are markers of neuroinflammation. Both wheel running and treadmill training have been shown to increase angiogenesis, therein total microvasculature, that could not only enable collateral blood supply to the peri-infarcted regions but also increase the availability of neuroprotective factors throughout the brain (Dirnagl, Becker, & Meisel, 2009; Swain et al., 2003). LG3 signaling could underlay some of these effects as DV, and therein LG3, has been shown to induce VEGF expression through the alpha 5 integrin receptor which would be expected to increase angiogenesis (Clarke et al., 2012). However, the specific biological processes that underlay the changes induced by exercise are still up for debate.

To investigate the progression of changes induced by exercise, Y. H. Ding et al. (2005) conducted a time course study to analyze the expression of ICAM-1 and the proinflammatory

cytokine tumor necrosis factor α (TNFα) over 3 weeks of treadmill training. Interestingly, they

found increased basal levels of ICAM-1 and TNFα in the brains of naïve mice, the magnitude of

which coincided with the cumulative amount of exercise (Y. H. Ding et al., 2005). These changes

could be indicative of ischemic pre-conditioning: the notion that applying an ischemic stimulus

at levels below the threshold for damage can augment the expression of neuroprotective factors

to be kept in reserve for a supra-threshold attack. Small repetitive intervals of cerebral ischemia

24 to 96 hours prior to MCAO have been shown to reduce infarct volume without inducing

neuronal death on its own (Shin et al., 2009; J. Zhang, Yang, Klaus, Koehler, & Huang, 2008). It is

possible that by expressing slightly elevated levels of ICAM-1 and TNFα prior to stroke, the

protective effects of inflammation are gained, that is to clear cellular debris and make space for

regeneration, without inducing a chronic, degenerative inflammatory environment. Moreover,

others have shown that inducing remote ischemic preconditioning (rIPC) at sites distal to organs

susceptible to ischemia such as the brain and the heart, also promotes protection. Inducing

remote ischemia on the hind limb by surgically occluding the femoral artery or applying a

tourniquet has been shown to reduce neurological deficits and cerebral infarct volume by 40%

(Hu, Lu, Zhang, Li, & Jiang, 2013; Ying Zhang et al., 2012). This method of rIPC has also been

mimicked in humans where blood pressure cuffs can be used to induce short intervals of

ischemia to a limb. Serum from these individuals was shown to reduce infarct size when used to

perfuse hearts isolated from experimental animals in an ex-vivo method of myocardial infarction

(Michelsen et al., 2012). Additionally, similar levels of protection were achieved from the serum

of human subjects that had undergone exercise tests such as swimming and running (Jean-St-

Michel et al., 2011). The buildup of lactate from anaerobic respiration after running suggests an

ischemic environment is associated with exercise (Ferreira et al., 2007). The findings from these

studies suggest that exercise could be method of achieving remote ischemic preconditioning through factors released into circulation.

There are numerous studies that have demonstrated the importance of an array of specific effectors of exercise such as endothelial nitric oxide synthase (Endres et al., 2003) and BDNF (Y. Ding et al., 2004) for example; none-the-less the search for a universal neuroprotective factor that has the potential for clinical translation has continued. An ideal molecule would encompass the capability for rapid, robust generation upon stimulation while exhibiting an appropriate spatial expression pattern. An extracellular matrix component such as perlecan possesses opportune localization as its proximity to circulation as well as the surrounding tissue enables bi-directional responses and actions. The neuroprotective effects of the DV and LG3 fragments have been demonstrated in several stroke models (Al-Ahmad et al., 2011; Bix et al., 2013; Lee et al., 2011). There is also evidence for the association of LG3 and exercise. Using surface-enhanced laser desorption/ionization (SELDI) mass spectrometry, Parker et al. (2012) was able to detect elevated levels of LG3 in the urine of individuals in labor-intensive mining occupations after their work shift. Elevated LG3 levels were specific to employees assigned to physically demanding occupations rather than those in more sedentary, support positions. It is possible that the increased LG3 present in urine is due to mechanical stress on blood vessels during physical exertion. However, the increased expression of BMP-1, a protease known to cleave LG3 (Gonzalez et al., 2005), in the current study suggests a more specific exercise induced cleavage. The concurrent increase in perlecan gene expression implies that not only is there the potential for increased liberation of LG3 by BMP-1, but an increased availability of substrate as well. While LG3 was not detected in serum in the current study, it is very likely that a more sensitive technique such as SELDI mass spectrometry would be able to detect a change in LG3 in exercised mice. Serum was drawn approximately 6 hours after the end of the dark cycle when

running wheel animals were typically most active. It is plausible that elevated LG3 levels are transient and by 6-hours post exercise serum LG3 will have returned to basal levels. It is also probable that more than 7 days of bursts of LG3 release are necessary to mimic the neuroprotection demonstrated in other preconditioning studies. The evidence of LG3 release with exercise and the significant neuroprotection afforded by DV/LG3 post MCAo suggest that it is a viable candidate as a molecular mediator of exercise neuroprotection though further study is required. The magnitude of the contribution of LG3 to neuroprotection can be evaluated using a model that is deficient of perlecan. Knockout of *Hspg2* induces embryonic lethality at E10 to E12 predominantly due to defective stress membrane development in the heart and brain (Arikawa-Hirasawa, Watanabe, Takami, Hassell, & Yamada, 1999). In the C1532Yneo transgenic mouse, a neomycin cassette was inserted into domain III of *Hspg2* (Rodgers, Sasaki, Aszodi, & Jacenko, 2007). These mice express low levels of a truncated form of perlecan and display a Schwartz-Jampel syndrome-like phenotype. As these C1532Yneo mice express reduced levels of perlecan, therein LG3, it would be predicted that they would not benefit from the neuroprotection afforded by LG3 release after exercise. Furthermore, if treating these mice prophylactically with recombinant LG3 reproduces MCAo infarct reduction to an extent similar of exercised wild type mice, it could signify that LG3 treatment can replicate some aspects of exercise preconditioning.

The effects of exercise on stroke are two-fold. Exercise ameliorates some of the modifiable risk factors for stroke and also affords neuroprotection itself. A focus on the vascular contributions in stroke could be particularly insightful for future neurodegenerative research as increased vascular resistance to ischemia can reduce numerous degenerative processes such as inflammation, edema, oxidative damage, and peripheral leukocyte infiltration to name a few. The identification of a molecular mediator of physical activity that can be used not only as a

biomarker but also processes broad neuroprotective effects can allow clinicians to supplement

the exercise regimes of patient populations that are at risk of stroke, especially those that may

not be capable of achieving an appropriate fitness level themselves.

Appendix I: List of Abbreviations

ACA	Anterior Cerebral Artery
ANOVA	Analysis of Variance
ATP	Adenosine Tri-Phosphate
BBB	Blood Brain Barrier
BCA	Bicinchoninic Acid
BMP-1	Bone Morphogenic Protein 1
BDNF	Brain-Derived Neurotrophic Factor
CBF	Cerebral Blood Flow
CCA	Common Carotid Artery
CDC	Center for Disease Control
CLDN5	Claudin-5
CO_2	Carbon Dioxide
CTSB	Cathepsin B
CTSL	Cathepsin L
DNA	Deoxyribonucleic Acid
DV	Domain V (of Perlecan)
ECM	Extracellular Matrix
ELVO	Emergent Large Vessel Occlusion
ERK	Extracellular Signal-Regulated Kinase
FAM	6-Carboxyfluorescein
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
HSPG2	Heparin Sulfate Proteoglycan 2 (Perlecan)
ICA	Internal Carotid Artery
ICAM	Intracellular Adhesion Molecule
IL-1β	Interleukin-1β
IL-10	Interleukin-10
ITGA5	Integrin Alpha 5
LG3	Laminin-like Globular Domain 3 (of Perlecan)
MCA	Middle Cerebral Artery
MCAo	Middle Cerebral Artery Occlusion
MGB	Minor Groove Binder
MMP	Matrix Metalloprotease
NGF	Nerve Growth Factor
NHLBI	National Heart, Lung and Blood Institute
NVU	Neurovascular Unit
OCLN	Occludin
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PPARGC1A	Peroxisome Proliferative Activated Receptor Gamma, Co-Activator 1α
PSD3	Post Stroke Day 3
RNA	Ribonucleic Acid

rRNA	Ribosomal RNA
RIPA	Radioimmunoprecipitation Assay
rIPC	Remote Ischemic Preconditioning
ROS	Reactive Oxygen Species
RPL38	60S Ribosomal Protein L38
RW	Running Wheel
SDS	Sodium Dodecyl Sulfate
Sed.	Sedentary
SELDI	Surface-Enhanced Laser Desorption/Ionization
TBI	Traumatic Brain Injury
TNFα	Tumor Necrosis Factor α
TTC	2,3-Triphenyltetrazolium Chloride
VCAM	Vascular Cell Adhesion Molecule
VEGF	Vascular Endothelial Growth Factor

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