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The Effects of Exercise Pattern and Intensity on the Expression of Flk-1 and Flt-1 Receptors in the Hippocampus and Cerebellum

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by

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ABSTRACT

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Aerobic exercise benefits the body and brain. In the brain, these benefits include neuroprotection and improved cognition. These exercise-induced changes are attributed in part to angiogenesis: the growth of new capillaries from preexisting vessels. One critical factor involved in the regulation of angiogenesis is VEGF and its receptors Flk-1 and Flt-1. Although exercise is generally found to be beneficial, there are wide variations in the exercise regimens used across experiments. This study standardized some of these variations. In this study, rats were either assigned to a voluntary wheel running or a forced wheel running exercise condition. Within each condition, animals ran at either a high (1,000 wheel revolutions) or low (500 wheel revolutions) intensity for up to 24 hours. Animals were removed from the running wheels when they reached their respective revolutions. In addition, there was a voluntary exercise group with unrestricted access to the running wheel for 24 hours. All animals were sacrificed at the end of the exercise regimens. Exercising animals were then compared to inactive controls, based on unbiased stereological quantification of Flk-1 and Flt-1 immunohistochemical labeling in the hippocampus and cerebellum. Two-way ANOVAs were completed with pattern (forced, voluntary, or inactive control) and intensity (high, low, or unrestricted) as independent variables and either Flt-1 or Flk-1 area fraction as the dependent variable. Findings indicated that regardless of intensity,

voluntary exercise, but not forced exercise, significantly increased Flk-1 and Flt-1 expression in the hippocampus. In the cerebellum there was no pattern effect, but low intensity exercise resulted in significantly greater Flk-1 expression compared to both high and unrestricted intensities. Interestingly, in the cerebellum, Flt-1 expression was significantly greater in inactive controls compared to both forced and voluntary exercise conditions.

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Introduction

Exercise produces neurovascular changes in the brain, which can be neuroprotective and support ischemic stroke recovery (Hoben et al., 2004; Austin, Ploughman, Glynn, & Corbett, 2014). Exercise may exert these protective effects through increased angiogenesis: the growth of new capillaries from preexisting blood vessels (Kerr & Swain, 2011; Nishijim & Soya, 2006; Black, Isaacs, Anderson, Alcantara, & Greenough, 1990). Increased angiogenesis, following ischemic stroke, highly correlates with improved functional outcomes in animal models and human patients (Hayashi, Noshita, Sugawara, & Chan, 2003; Krupinski, Klauza, Kumar, Kumar, & Wang, 1994). Understanding how exercise pattern and intensity influence angiogenesis could lead to improved exercise rehabilitation programs following ischemic stroke (Hoben et al., 2004; Austin et al., 2014). Presumably, the exercise pattern and intensity that produces the most robust angiogenic changes early in training would likely be the most beneficial for patients recovering from an ischemic insult.

Angiogenesis is studied using voluntary and forced exercise patterns (Lou, Liu, Chang, & Chen, 2008; Kerr, Steuer, Pochtarev, & Swain, 2010). Voluntary wheel running allows the rodent complete control over the exercise, which typically involves rapid running speeds for short durations (Leasure & Jones, 2008). In forced treadmill exercise, the researcher controls the exercise, which generally requires the rodent to run at a slower speed, for a set duration with no breaks (Leasure & Jones, 2008). Although both patterns induce neurovascular changes, the findings are inconsistent. Some studies suggest voluntary exercise (Ke, Yip, Li, Zheng, & Tong, 2011) and others report forced exercise (Leasure & Jones, 2008) produce more robust neurovascular changes. Conflicting findings may be explained by differences in exercise intensity. For example, after seven days of forced exercise, at a slower speed, rodents in the low

intensity group displayed increased neurogenesis and neurovascular growth factor expression compared to the faster, high intensity forced exercise group (Lou et al., 2008). Few studies have investigated the effects of exercise intensity using voluntary exercise. However, performance on the Morris water maze improves when access to voluntary running wheels was unrestricted, rather than restricted (van Praag et al., 2007). Furthermore, most studies investigating the differential effects of exercise pattern focus on neuronal, rather than vascular changes (Lou et al., 2008; Leasure & Jones, 2008; Ke et al., 2011), and few studies quantify *acute* angiogenic changes that occur during exercise. Most experiments have had animals run for longer durations, ranging from 1-8 weeks, before quantifying neurovascular changes (Lou et al., 2008; Leasure & Jones, 2008; Ke et al., 2011; Kerr & Swain, 2011). However, these angiogenic changes begin within hours of exercise onset (Kerr et al., 2011). This study aims to understand how exercise intensity and pattern affects the expression of Flk-1 and Flt-1 receptors in the hippocampus and cerebellum after an acute bout of exercise. Flk-1 and Flt-1 are high affinity receptors for vascular endothelial growth factor (VEGF), a key angiogenic factor (Hoben et al., 2004).

Literature Review

Angiogenesis

All eukaryotic cells require a source of oxygen and glucose to survive. Without a sufficient supply of these nutrients cells cannot meet metabolic demands, resulting in cellular dysfunction and death (Semenza, 2007). Oxygen and glucose delivery is provided by the vascular system, which by design ensures all cells are within proximity of an adequate supply of nutrients (Iyer et al., 1998; Carmeliet, 2005; Semenza, 2007). The vascular system is maintained, in part, by angiogenesis: the growth of new capillaries from preexisting blood vessels (Carmeliet, 2005). Initially, angiogenesis was viewed as a phenomenon exclusive to development. However,

it is now well established that angiogenic changes occur in response to a variety of pathological and non-pathological conditions in the body and brain (Swain et al., 2012). For example, angiogenesis is upregulated in the periphery during periods of wound healing, menstruation and ovulation, and organ regrowth (Otrock Mahfouz, Makarem, & Shamseddine, 2007; Carmeliet, 2005). Additionally, it is involved in the development of pathological conditions such as tumor growth, diabetes, and rheumatoid arthritis (Carmeliet, 2005). In the brain, angiogenesis is upregulated in response to ischemic insults and other experiences, including environmental enrichment and exercise (Yu et al., 2014; Kerr & Swain, 2011; Swain et al., 2003).

Angiogenesis is highly regulated by a complex system of transcription and growth factors. Active angiogenesis only occurs for brief periods of time and is then rapidly inhibited in effort to prevent vascular overgrowth (Otrock et al., 2007). Angiogenesis is initiated when the local expression of pro-angiogenic factors increase, relative to anti-angiogenic factors (Otrock et al., 2007; Kalluri, 2003). Elevations in pro-angiogenic factors trigger endothelial cell proliferation and migration. The angiogenic process first requires destabilization of the target vessel. This involves a selective degradation of the basement membrane and remodeling of the extracellular matrix by enzymes primarily from the matrix metalloproteinase (MMP) system (Krock, Skuli, & Celeste Simon, 2011; Ben-Yosef, Miller, Shapiro, & Lahat, 2005; Rundhaug, 2005). The MMP system is important for the detachment of pericytes, release of growth factors sequestered in the extracellular matrix and basement membrane, cleavage of endothelial cell to cell adhesions, liberation of endothelial cells, and the exposure of integrin binding sites (Rundhaug, 2005; Kalluri, 2003). Together, these actions increase the permeability of the target vessel and create space for the new vessel to grow (Rundhaug, 2005). One key growth factor involved in the destabilization process is angiopoiten-2 (Ang-2). During normoxia Tie-2

receptors, located on the surface of endothelial cells, primarily bind angiopoieten-1 (Ang-1). This signal stabilizes the vessel. During periods of hypoxia, levels of Ang-2 increase. Ang-2 binding to the Tie-2 receptor blocks Ang-1 /Tie-2 signaling and instead signals pericytes to pull away from the capillary, destabilizing the vessel (Yuan, Khankin, Karumanchi, & Parikh, 2009; Bogdanovic, Nguyen, & Dumont, 2006). Endothelial cells are nonreactive when attached to the basement membrane; however, when released they are free to interact with pro-angiogenic factors such as vascular endothelial growth factor (VEGF) (Rundhaug, 2005).

After the vessel is destabilized, proliferation and migration begin. The new vessel is composed of a network of stalk and tip cells. Stalk cells trail behind tip cells and are critical for proliferation. They are involved in generating the trunk of the new vessel that maintains attachment to the parent vessel. VEGF binding to receptor fetal liver kinase-1 (Flk-1) generates this proliferative response in stalk cells. In contrast, VEGF signaling in tip cells leads the migration of the new vessel, as tip cells extend filopodia and move in response to VEGF concentrations (Krock et al., 2011; Gerhardt et al., 2003). The filopodia on tip cells also release proteolytic enzymes involved in the breakdown of material in the extracellular matrix obstructing the pathway of the newly forming vessel (Adair & Montani, 2010). Flk-1 receptors are abundant on these filopodia and Flk-1 signaling is necessary for the migration of tip cells. When Flk-1 receptors were neutralized, using a neutralizing Flk-1 specific antibody, tip cell filopodia retracted and migration was inhibited (Gerhardt et al., 2003). The degree of Flk-1 expression on stalk and tips cells is not significantly different; however, the binding of VEGF to Flk-1 on these two endothelial cell types activates distinct cellular responses. One response, generated by the stalk cells, is critical to proliferation and the other, generated by the tip cells, is necessary for migration (Gerhardt et al., 2003). Stalk cells proliferate and trail behind the tip cells, as the tip

cells migrate towards pro-angiogenic stimuli. These two cell types make up the new capillary sprout (Adair & Montani, 2010).

When tip cells from two or more capillary sprouts meet, at the location where proangiogenic factors are highly expressed, they fuse forming a continuous vessel. The new vessel is arranged in a tubular structure by integrins and stabilized by the recruitment of pericytes. Newly produced vessels decrease intercapillary diffusional distance, improving the delivery of oxygen and glucose to nearby cells (Swain et al., 2012). If there is not an upregulation of pro-angiogenic factors, the newly developing capillaries will undergo apoptotic regression (LaManna, Chavez, & Pichiule, 2004; Pichiule & LaManna, 2002).

Hypoxia

In the brain, hypoxia is a key initiator of angiogenesis. Hypoxic states are caused by a range of conditions and experiences including ischemic insult, exercise, and environmental enrichment (Yu et al., 2014; Swain & Kerr, 2011; Mennel, El-Abhar, Schilling, Bausch, & Krieglstein, 2000). To function, the brain requires about 20% of the total volume of oxygen consumed by the body (Rolfe & Brown, 1997). Oxygen delivery to the brain is tightly regulated and changes in response to local demand. When oxygen levels deplete, either due to increased metabolic activity and/or hypoxia, the brain rapidly undergoes neurochemical changes aimed to promote neuronal survival (Harten, Ashcroft, & Maxwell, 2010). These changes, in part, involve the upregulation of angiogenic factors, and the degree to which the brain exhibits these changes depends on the duration and degree of hypoxia (Marti et al., 2000).

Degree. Although the degree of hypoxia an organism may experience exists on a continuum, it is generally divided into three categories: mild, moderate, and severe. First, moderate hypoxia will be discussed followed by severe and mild. Moderate states of hypoxia

typically do not physically damage tissue; however, they may produce temporary deficits in synaptic transmission. Hippocampal slices exposed to moderate hypoxia (10% oxygen) for two consecutive eight hours periods did not display increased neuronal cell death rates when compared to controls. However, there was a transient decrease in synaptic transmission in hippocampal slices exposed to moderate hypoxia. Transmission returned to normal 30 minutes post-exposure (Weber et al., 2005). In an earlier study, hippocampal slices exposed to moderate hypoxia also exhibited a loss in synaptic transmission, and, although transmission eventually recovered, synapses remained hyperexcitable for the duration of the experiment (Schiff & Somjen, 1981).

Moderate hypoxia may produce temporary impairments; however, it can also be neuroprotective. Moderate hypoxia (10% oxygen) protected against neuronal damage in the hippocampus induced by severe hypoxia (3% oxygen), if animals were successively exposed to moderate degrees of hypoxia prior to exposure to severe hypoxia (Gorgias et al., 1996). Thus, moderate hypoxia may produce transient impairments, but animals rapidly recover from these impairments with no notable behavioral deficits and are protected from the damaging effects of severe hypoxia (Schiff & Somjen, 1981; Gorgias et al., 1996).

Severe forms of hypoxia result in extensive neuronal damage and necrotic death in affected brain regions (Rybnikova et al., 2005; Mennel et al., 2000). To induce severe hypoxia, Rybnikova et al. (2005) exposed rodents to extreme hypobaric hypoxia (5% oxygen) for three hours and found profound morphological damage to vulnerable neurons in the hippocampus and neocortex. Three days after exposure to severe hypoxia, the neurons visualized displayed characteristics of necrosis and apoptosis, including severe chromatolysis, pyknosis, and

cytoplasmic vacuolization (Rybnikova et al., 2005). Seven days after exposure the bout of severe hypoxia, there was a 30% loss of CA1 neurons in the hippocampus (Rybnikova et al., 2005).

Rather than exposing animals to a hypoxic environment, permanent cerebral artery occlusion can be used to induce severe hypoxia. Mennel and colleagues (2000) permanently occluded the middle cerebral artery (MCA) in mice and sacrificed animals after 30 or 60 days. At both time points, neurons exhibited signs of necrosis in the ischemic core, and neurons surrounding the core in the penumbra were apoptotic. Gliosis was also exhibited, particularly in the astrocytes (Mennel et al., 2000). This neurological damage is accompanied by behavioral deficits. For example, in addition to neuronal damage in the hippocampus and cortex following exposure to severe hypoxia, animals displayed deficits in spatial learning on the Morris water maze (MWM) (Simonova, Sterbova, Brozek, Komarek, & Sykova, 2003) and impaired performance on a passive avoidance (PA) task (Rybnikova et al., 2005). The effects of severe hypoxia are adverse, and always evident is some degree of irreversible necrotic and apoptotic cell death. However, it is important to note the brain will undergo neurovascular changes aimed to ameliorate the damage induced by severe hypoxia. For example, Marti et al. (2000) found VEGF and VEGF receptors were upregulated in the ischemic border and core following MCA occlusion. Furthermore, there was an increase in the number of new vessels formed near the infarct border between 48-72 hours post occlusion (Marti et al., 2000). Unfortunately, these angiogenic changes are not enough to fully prevent and repair the damage induced by severe hypoxia. This is an important distinction between severe and milder states of hypoxia.

The energy supply to the brain is impaired during bouts of severe hypoxia, and this has deleterious consequences. In contrast, following exposure to mild hypoxia, levels of ATP in the brain are near normal (Gibson, Pulsenelli, Blass, & Duffy, 1981), and mild hypoxia elicits robust

neuroprotective effects. Preconditioning using conditions of mild hypobaric hypoxia decreases the severity of neuronal damage and eliminates performance impairments on a passive avoidance task typically presented after severe hypoxia exposure (Rybnikova et al., 2005).

Mild states of hypoxia exert these protective effects, in part, through the proliferation and survival of neurons. Following exposures to mild hypoxia, there was an overexpression of neural proliferation signals, including proliferating cell nuclear antigen, Rb, and Bcl-2. In addition, there was also a suppression of p53, b21, caspases, and Bax, all signals and markers of apoptotic cell death (Bossenmeyer-Pourie et al., 2002). Santilli and colleagues (2010) investigated the effects of mild hypoxia on the proliferation of neuronal stem cells and found that mild hypoxia yields a higher percentage of Ki67 positively labeled cells compared to control levels. Ki67 is a marker of neuronal stem cell proliferation (neurogenesis) (Santilli et al., 2010). Similarly, intermittent exposure to mild hypoxia for 14 days enhances neurogenesis in the dentate gyrus and subventricular zone, as indicated by $BrdU^{+}$, co-localized neuronal marker NeuN, labeling and elevations in brain derived neurotrophic factor (BDNF). No neurotoxic effects were detected (Zhu et. al., 2010). Taken together, these studies demonstrate mild hypoxia promotes neurogenesis and prevents the death of existing neurons.

Not only does mild hypoxia promote neurogenesis and neuronal survival, but it also promotes angiogenesis. Animals exposed to mild normobaric hypoxia for 49 days show significant increases in vessel density in the cerebral cortex, hippocampus, and striatum. Increased vessel density was also detected in the cerebellum and medulla oblongata (Patt, Sampaolo, Theallier-Janko, Tscharikin, Cervos-Navarro, 1997). Numerous other studies have reported similar findings (Harik, Hritz, LaManna, 1995; Harik et al., 1996; LaManna et al., 2004; Masamoto et al., 2013). These angiogenic changes that occur in response to mild hypoxia are

rapid and robust. Capillary density increases have been detected after one week of hypoxia exposure and density nearly doubles after three weeks of exposure (Lario & LaManna, 1997).

Advantageous changes in behavior parallel the neurovascular changes that follow mild hypoxia. Exposure to mild hypoxia elicited antidepressant effects in adult rats exposed to chronic mild stress (Zhu et al., 2010) and decreased anxiety-like behavior in adult mice (Leconte et al., 2012). Additionally, unlike animals exposed to severe hypoxia, which display behavioral performance deficits, animals exposed to mild hypoxia exhibited enhanced behavioral performance on a variety of tasks. Mice exposed to mild (16% oxygen), intermittent hypoxia from birth to four weeks of age demonstrated enhanced performance on the MWM and eight-arm radial arm maze. This improvement lasted until animals reached post-natal day 85-89. Unlike animals exposed to mild hypoxia, animals exposed to moderate hypoxia (10.8% oxygen) did not display these lasting behavioral improvements (Zhang, Chen, Du, Chen, & Zhu, 2005). Mild hypoxia is unique in comparison to severe and moderate hypoxia, in that it is *both* neuroprotective and enhances performance on behavioral tasks. These positive behavioral effects are likely due, at least partially, to increases in neurogenesis and angiogenesis.

Duration. States of hypoxia not only vary in degree, but also in the duration of exposure. Duration is typically categorized as either acute or chronic. Acute, or transient, hypoxia involves one relatively brief bout of hypoxia. The interval of exposure can range from minutes to hours or a few days (Xu & LaManna, 2006). When periods of hypoxia are brief, cerebral vessels undergo vasodilation in effort to increase blood flow and hence oxygen delivery (Masamoto & Tanishita, 2009). When anesthetized rats were exposed to gradual reductions in oxygen levels, there were increases in cerebral blood flow in the neocortex, striatum, cerebellum, and corpus callosum in comparison to control animals. Additionally, increases in blood flow were proportional to the

severity, or degree, of the hypoxic condition, as measured using contrast magnetic resonance imaging (MRI) (Julien-Dolbec et al., 2002). Cerebral blood flow increases as much as twofold in response to hypoxia (Xu & LaManna, 2006).

Chronic hypoxia typically refers to hypoxia exposures that persist for several days, to weeks or months (Xu & LaManna, 2006). A state of chronic hypoxia can either refer to one, continuous exposure, or several shorter, repeated exposures, lasting several days or more. When hypoxia is chronic, or prolonged, the brain must further adapt to this condition and capillary density increases (Harik et al., 1995). Increases in capillary density are due to both the dilation of preexisting vessels, which begins during the acute phase, and angiogenesis (Harik et al., 1995). Mice placed in a hypobaric chamber for 23 hours per day, for four weeks, displayed a 25% increase in cortical volume in comparison to control animals. Angiogenesis contributed to this volumetric change. In the cerebellum, capillaries in mice exposed to hypoxia were elongated and more tortuous and dilated. Similar changes were found in the granular layer in the corpus striatum. Remodeling of the capillary plexus was also detected in the hippocampus, and capillaries were elongated in most layers and areas of the hippocampus, including CA1, CA3, and DG. There were also increases in capillary density in the motor cortex, along with increased elongation and tortuosity (Boero, Ascher, Arregui, Rovainen, & Woolsey, 1999).

Factors involved in the angiogenic process are elevated within hours of hypoxia exposure; however, actual increases in new vessels are not detectable until 1-2 weeks of chronic hypoxia exposure. After mice were subject to hypobaric hypoxia for one week, there was a significant increase in cerebral microvessel protein. There was no further increase in this protein after two or three weeks of exposure. Increases in this protein are indicative of an initial vasodilation response to hypoxia. In contrast, microvessel DNA yield did not increase after one

week of hypoxia but was significantly elevated after two weeks and continued to increase by three weeks of hypoxia exposure. This increase in microvessel DNA marks endothelial cell division, a hallmark of angiogenesis (Harik et al., 1995). Another study observed similar changes using two-photon microscopy. After inserting a closed cranial window over the sensory motor cortex, animals were housed in a hypoxic room (8-9% oxygen) for one or two weeks. Images were collected pre-hypoxia and weekly after hypoxia treatment began. After both one and two weeks of exposure there was an increase in the sprouting of new blood vessels from preexisting capillaries at a rate of 15 new vessels per mm^3. This was accessed by collecting and comparing images taken from identical locations in the motor cortex week to week. The highest emergence rate was found in the cortical depths of 100-200μm, indicating no spatial uniformity among the cortical layers (Masamoto et al., 2013).

In sum, exposure to chronic hypoxia results in robust angiogenesis (Harik et al., 1995; Masamoto et al., 2013). Elevations in angiogenic factors are detected within hours of exposure to hypoxia and increased vessel number is detected after 1-2 weeks of chronic exposure (Harik et al., 1995 Boero et al., 1999). Acute exposure only relies on the dilation of vessels, rather than angiogenesis, to compensate for decreased oxygen levels (Xu & LaManna, 2006). Angiogenic growth requires days to develop, whereas vasodilation occurs rapidly. In regard to the degree, only mild conditions of hypoxia trigger strong increases in angiogenesis (Harik et al., 1995; Harik et al., 1996; LaManna et al., 2004; Masamoto et al., 2013). Moderate and severe hypoxias promote these angiogenic changes to an extent, but numerous adverse effects accompany them (Rybnikova et al., 2005). Therefore, experimental methods used to study angiogenesis often instate mild, chronic exposures to hypoxia. One leading method used to produce a state of mild chronic hypoxia is aerobic exercise.

Exercise

Aerobic exercise produces numerous benefits in the brain and periphery. These benefits include decreased risks of developing obesity (Slentz et al., 2004), breast and colon cancer (Bernstein, Henderson, Hanisch, Sullivan-Halley, & Ross, 1994; Lund Nilsen & Vatten, 2001), cardiovascular disease (Oguma & Shinoda-Tagawa, 2004), type II diabetes (Sigal, Kenny, Wasserman, & Castaneda-Sceppa, 2004), osteoporosis (Gutin & Kasper, 1992), and mental illnesses including depression and anxiety (Ströhle, 2009). In addition to these substantial health benefits, exercise strongly influences brain structure, function, and cognition in both human and non-human animals (Swain et al., 2012). Two structural changes elicited by aerobic exercise include angiogenesis and neurogenesis. Aerobic exercise induces neurogenesis in the hippocampus (Deng, Aimone, & Gage, 2010), and angiogenesis has been reported in the hippocampus, cerebellum, and motor cortex following aerobic exercise (Swain et al., 2012; Isaacs, Anderson, Alcantara, Black, & Greenough, 1992; Swain et al., 2003; Kerr et al., 2010).

Hippocampus. The hippocampus houses the subgranular zone, a region within the dentate gyrus. The subgranular zone is one of two regions of the brain that contains neuronal progenitor cells (NPCs) throughout adulthood. The other region containing NPCs is the subventricular zone of the lateral ventricle. New neurons born in the subgranular zone proliferate, differentiate, and migrate to locations within the local neural network of the dentate gyrus where they are integrated into the existing circuits. This process, termed neurogenesis, is facilitated by exercise (Deng et al., 2010). Voluntary wheel running nearly doubles the number of $BrdU^+$ neurons in the dentate gyrus after four weeks (van Praag, Kempermann, & Gage, 1999). These increases in neuronal cell proliferation following aerobic exercise in the hippocampus are well established and highly robust, and neurogenesis following exercise occurs

in both young and aged rodents (van Praag, Shubert, Zhao, & Gage, 2005; van Praag et al., 1999; Allen et al., 2001). Furthermore, the distance run during voluntary exercise paradigms is positively correlated with cell proliferation and survival (Allen et al., 2001).

A vascular supply is required to support these newly birthed neurons produced following exercise. Interestingly, proliferative clusters of neuronal cells in the subgranular zone are assembled around capillaries. This grouping, termed the neurovascular niche, suggests neurogenesis occurs within an environment that involves vascular remodeling and recruitment (Palmer, Willhoite, & Gage, 2000). In addition, this reveals that neurogenesis and angiogenesis are tightly linked in the subgranular zone, possibly by codependent mechanisms (Palmer et al., 2000). Findings following this influential paper by Palmer and colleagues (2000) continue to support this assertion. Angiogenesis is also upregulated in the hippocampus following aerobic exercise, and this upregulation, at least in part, is necessary to meet the metabolic requirements of newly birthed neurons (Kerr et al., 2010; Fabel et al., 2003; Van der Borght et al., 2009). For example, after three weeks of wheel running, there was a significant increase in Glut-1 positive vessel density, and this angiogenic change correlated with neuronal proliferation in the dentate gyrus. Neuronal proliferation was increased after three days of exercise and was significantly elevated after 10 days of exercise (Van der Borght et al., 2009).

Motor Cortex and Cerebellum. Aerobic exercise also increases angiogenesis in brain regions involved in motor movement such as the cerebellum and motor cortex. In the first experiment aimed to dissociate the effects of learning and motor activity on vascular changes, Black et al. (1990) found rats that exercised on a treadmill or running wheel for 30 days displayed increased numbers of newly formed blood vessels in the molecular layer of the paramedian lobule, compared to controls and animals that learned an acrobatic task. Animals

completing the learning task displayed increased syntaptogenesis, rather than angiogenesis. The paramedian lobule was selected because it is online during forelimb movements. (Black et al., 1990). Thirty days of exercise also decreased vascular diffusion distance in the molecular layer of the cerebellar paramedian lobule (Isaacs et al., 1992). A more recent study found these angiogenic changes in the paramedian lobule occur as early as after 24 hours of voluntary wheel running, as measured by a Western blot for CD61 integren (Kerr & Swain, 2012).

Thirty days of exercise produces similar effects in the motor cortex. Rats that engaged in voluntary wheel running for 30 days displayed increased blood vessel density in the fourth layer of the forelimb region of the motor cortex (Kleim, Cooper, & VandenBerg, 2002). Using flow-alternating inversion recovery (FAIR) functional magnetic resonance imaging (fMRI) Swain and colleges (2003) found that rats in the voluntary wheel running group showed increased capillary perfusion in the forelimb region of the motor cortex, compared to inactive control animals. Additionally, completing immunohistochemistry for the CD61 integrin, which is only expressed on developing capillaries, suggested angiogenesis had robustly increased in the motor cortex after 30 days of voluntary wheel running (Swain et al., 2003).

Exercise and Angiogenesis

Angiogenesis is triggered in several brain regions in response to aerobic exercise (Swain et al., 2003; Kerr & Swain, 2011; Van der Borght et al., 2009). One key factor initiating this angiogenic cascade is the mild state of hypoxia produced by aerobic exercise. Exercise increases metabolic demands, and thus creates a mild, often chronic state of hypoxia. Angiogenesis is upregulated, in effort to compensate for this deficit and support new neuronal growth that takes place in response to exercise in the hippocampus.

Apoptosis. One indication that aerobic exercise creates a state of hypoxia is signs of apoptotic cell death, which occurs rapidly after the onset of aerobic exercise. Kerr and Swain (2011) found elevations of cleaved caspase-3, an apoptotic signal, after 12 hours of voluntary wheel running in both the hippocampus and cerebellum. Levels were significantly elevated in both structures after 24 hours of exercise. At time points after 24 hours of exercise, expression returned to baseline levels (Kerr & Swain, 2011). There may not be enough oxygen and glucose readily available to meet the increased metabolic demands exercise places on these brain regions. Thus exercise initially produces mild, apoptotic cell death. This apoptotic event may serve as a signal for angiogenesis upregulation. Some evidence exists for this assertion, as in Kerr and Swain's (2011) experiment in which angiogenesis, detected between 24-48 hours of voluntary exercise, immediately preceded increased apoptosis (Kerr & Swain, 2011).

HIF-1α. A second indicator of mild hypoxia being associated with aerobic exercise is elevations in hypoxia inducible factor-1α (HIF-1α), which occur following aerobic exercise (Berggren, 2013). HIF-1 is heterodimeric, with both an α-subunit and β-subunit; however, it is the HIF-1α subunit that plays a key role in oxygen regulation. HIF-1α is a transcription factor uniquely involved in sensing and responding to changes in oxygen tension (Iyer et al., 1998). HIF-1α itself does not directly sense oxygen tension, rather proline hydroxylase domain (PHD) containing proteins and factor inhibiting HIF (FIH) proteins detect oxygen changes and post-translationally modify HIF-1α by hydroxylating amino acid residues (Forsythe et al., 1996; Virtue & Vidal-Puig, 2011).

Under conditions of normoxia, HIF-1α levels are regulated through protein degradation. HIF-1α is modified by prolyl hydroxylase 1-3 (PHD 1-3) as it is produced, and this modification allows for the binding of von Hippel-Lindau (VHL) E3 ubiquitin ligase complex to HIF-1α. HIF-

1α is polyubiquitinated and then degraded by the 26S proteasome (Chen, Endler, & Shibasaki, 2009; Iyer et al., 1998; Ohno et al., 2012; Jaakkola et al., 2001; Krock et al., 2011). However, when oxygen levels are reduced, PHD activities are inhibited, allowing for the stabilization and increase in HIF-1α protein. HIF-1α then translocates to the nucleus where it facilitates the transcription of numerous genes involved in adaptation to hypoxia (Ohno et al., 2012; Iyer et al., 1998). Over 100 genes have been identified as HIF-1α targets (Ohno et al., 2012). These include genes involved in glycolysis, erythropoiesis, and angiogenesis (Hoppeler, Vogt, Weibel, & Fluck, 2003).

PHDs are not the only regulators of HIF-1α. It is also regulated by FIHs. Instead of degrading HIF-1α, the FIHs modify the way HIF-1α interacts with transcriptional coactivators. FIHs not only prevent HIF-1α expression during normoxia, but also they are active during periods of hypoxia, when PHDs are completely inactive and HIF is stable. Thus, FIHs seem to be more sensitive to oxygen tension in comparison to PHDs and serve as a regulator of HIF expression during both periods of normoxia and hypoxia (Mahon, Hirota, & Semenza, 2001; Lando et al., 2002). It is also important to note that HIF-1α may not be solely be regulated though oxygen tension. Oxidative stress, acidosis, and heat may all influence the stabilization of HIF-1α (Cooper, Radom-Aizik, Schwindt, & Zalivar, 2007; Krock et al., 2000). For example, an increase in reactive oxygen species (ROS) may decrease the availability of free iron, which would also inhibit PHD activity and result in increased HIF-1α accumulation (Cash, Pam, & Simon, 2007).

HIF-1α promotes changes that aid in both short- and long-term adaptions to hypoxia. Short-term changes include increases in inducible nitric oxide synthase (iNOS). This is a vasodilatory enzyme and produces nitric oxide (NO), and NO has a role in relaxing vascular

smooth muscle cells. This relaxation results in increased in blood flow (Jung, Palmer, Zhou, & Johns, 2000). In addition to providing increased blood flow, HIF-1α also lowers oxygen demand by increasing glycolysis. Specifically, HIF-1α affects this process by inducing glycolytic enzymes, increasing glucose uptake by increasing glucose transporter-1 (GLUT-1), and preventing mitochondrial respiration through increases in pyruvate dehydrogenase kinase (PDK1) (Kim, Tchernyshyov, Semenza, & Dang, 2006; Ryan, Lo, & Johnson, 1998; Iyer et al., 1998). HIF-1α also further limits oxygen demand by decreasing cell proliferation via the upregulation of cyclin-dependent kinase inhibitors p21 and p27 (Carmeliet et al., 1998).

Long-term adaptions to mild hypoxia regulated by HIF-1α primarily include stimulating angiogenesis (Krock et al., 2011). HIF-1α regulates numerous pro-angiogenic genes and primary targets include VEGF, Ang-1, Ang-2, platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFBF), erythropoietin, and monocyte chemoattractant protein-1 (MCP-1). These targets support angiogenesis through increasing vascular permeability, and the proliferation, sprouting, migration, adhesion, and tube formation of new vessels (Krock et al., 2011). Although all of these factors have important roles, VEGF is the most potent regulator of angiogenesis during development and adulthood (Carmeliet et al., 1996; Marti et al., 2000).

Several studies have investigated the effects of exercise on HIF-1α expression in skeletal muscles. When rats were treated with one hour of sciatic nerve electrical stimulation, in an effort to mimic the effects of exercise, there was a six-fold increase in HIF-1α protein levels with maximal electrical stimulation (8V) in the gastrocnemius muscle (Tang et al., 2004). Additionally, one bout of exercise for 45 minutes increased HIF-1α protein in the vastus lateralis muscle, and HIF-1α remained elevated for 360 minutes post exercise (Ameln et al., 2005). When the gene encoding for HIF-1α in the skeletal muscle was deleted via genetic knockout, there was

a significant increase in muscle damage in the knockout mice after repeated exercise trials, in comparison to wild-type animals. Animals with the knockout also exercised significantly less (Mason et al., 2004). Few studies have investigated the effects of exercise on HIF-1α in the brain; however, similar elevations of HIF-1α in response to exercise have been documented. HIF-1α was significantly elevated in animals that engaged in treadmill exercise at 30 m/min, compared to inactive controls, after three weeks of exercise (Kinni et al., 2011; Berggren, 2013).

VEGF. One critical proangiogenic factor regulated by HIF-1α is vascular endothelial growth factor (VEGF) (Hoben et al., 2004). VEGF mRNA expression is upregulated through exposure to low levels of oxygen, and it is well established that HIF-1α is a critical mediator of VEGF expression in response to hypoxia (Ferrara, Gerber, & LeCouter, 2003). Mice lacking the HIF-1 gene die midgestation and display deficits in angiogenesis and decreased levels of VEGF gene expression (Kotch, Iyer, Laughner, & Semenza, 1999). However, even without HIF-1 animals still exhibit some response to hypoxia, which indicates there are other, HIF-1α independent, mechanisms involved in producing VEGF mRNA (Kotch et al., 1999).

VEGF is one of the most potent endothelial cell specific mitogens and regulates angiogenesis during development and adulthood, in both the brain and periphery (Ferrara et al., 2003; Carmeliet et al., 1996; Marti et al., 2000). VEGF promotes the survival of new and existing endothelial cells and increases the permeability of the vasculature (Hayashi, Abe, Suzuki, & Itoyama, 1997; Ferrara et al., 2003). Delivery of VEGF stimulates the recruitment of endothelial cells to hypoxic regions and stimulates their division (Conway, Collen, & Carmeliet, 2001). During development, lacking a single VEGF allele led to abnormalities in the formation of blood vessels that were lethal by mid-gestation (Carmeliet et al., 1996). VEGF is part of a gene family that includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F, and

placental growth factor (PLGF) (Hoben et al., 2004; Ferrara et al., 2003). VEGF-A and VEGF are often used interchangeably in the literature and VEGF-A is the key regulator of angiogenesis in a variety of tissue types. The main focus will be on VEGF-A, or VEGF; however, it is important to note that the other homologs exhibit VEGF-like activities (Ferrara et al., 2003). For example, VEGF-C and VEGF-D are involved in lymphatic angiogenesis (Karkkainen, Makinene, & Alitalo, 2002).

VEGF is rapidly transcribed following hypoxic or ischemic events. There is a strong temporal correlation between VEGF expression and hypoxia (Hayashi et al., 1997; Marti e al., 2000). Activation of VEGF has been detected in the penumbra following ischemia, and expression is detected shortly after the ischemic event (Hayashi et al., 1997; Marti et al., 2000). For example, VEGF protein rapidly increased in ischemic border zones between 6-24 hours after a permanent MCA occlusion in rodents (Marti et al., 2000). After a transient MCA occlusion, VEGF mRNA elevations in cortical neurons were detected after one hour of reperfusion, peaked after three hours, and slowly decreased thereafter (Hayashi et al., 1997). Furthermore, angiogenesis temporally follows VEGF elevations post-ischemic insults. Increased endothelial cell proliferation has been detected one day after the induction of ischemia, with a significant increase in blood vessel number after one day (Hayashi, Noshita, Sugawara, & Chan, 2003). This increase in blood vessel number occurs after VEGF mRNA and protein are elevated (Hayashi et al., 1997; Marti et al., 2000). It is also important to note that VEGF does not only produce new vessels in the ischemic region; its angiogenic function also saves existing neurons. For example VEGF decreased the number of TUNEL-positive neurons, a marker of apoptosis, in the ischemic penumbra (Kaya et al., 2005).

In addition to VEGF elevations temporally following MCA occlusions, the infusion of VEGF during reperfusion promotes recovery in damaged regions. Following a 90-minute focal cerebral ischemia, rats either received an intracerebroventricular infusion of VEGF or no infusion of VEGF on reperfusion days 1-3. Rats that received VEGF infusions displayed improved neurological performance, decreased infarct size, and increases in angiogenesis in the ischemic penumbra (Sun et al., 2003). VEGF exerts similar effects when rodents receive infusions after occlusion of the right carotid artery. Delivery of 10 or 20 ng of VEGF intracerebroventricularly five minutes after reperfusion reduced brain weight loss and gross infarct size (Feng, Rhodes, & Bhatti, 2008). It is likely that this reduced infarct size is partially due to angiogenesis, which increases both vessel density and promotes neuronal survival.

Like in cases of cerebral ischemia which create a state of hypoxia, VEGF functions similarly in more mild states of hypoxia induced by aerobic exercise. It is well established that VEGF has a critical role in aerobic exercise induced angiogenesis in both the brain and periphery in human and non-human animals (Swain et al., 2003; Kerr & Swain, 2011; Van der Borght et al., 2009; Amaral, Papaneck, & Greene, 2001). In rodents, three days of treadmill exercise resulted in significant increases in VEGF protein expression in exercising animals in the tibialis anterior and gastrocnemius muscles. This elevation occurred in concurrence with increased blood vessel density (Amaral et al., 2001). Using a more intense and lasting exercise paradigm, Lloyd, Prior, Yang, and Terjung (2003) found similar effects. In this experiment, animals were required to run on the treadmill four times per day, for either 1, 3, 8, 12, 18, or 24 days. Exercise initially involved walking at slower speeds but was gradually ramped up until animals were running at a speed of 20-25 m/min. For each bout of exercise, animals ran until they displayed signs of fatigue, which included a hopping gait. VEGF mRNA was significantly increased in the white

gastrocnemius muscles after one day of exercise and was significantly elevated in the red gastrocnemius and soleus muscles after 2-3 days. VEGF mRNA was increased 3-6 fold, depending on the muscle. Increases in capillary density were detectable after 12 days of exercise when VEGF mRNA levels returned to control levels (Lloyd et al., 2003). This temporal relationship suggests VEGF is important for angiogenic changes in the skeletal muscle, and it follows the patterns of expression detected in cases of ischemia induced by MCA occlusions. VEGF levels increase prior to detectable changes in vessel density (Hayashi et al., 1997; Marti et al., 2000).

To further understand the role of VEGF in skeletal muscle vascular changes following exercise, Olfert et al. (2009) generated mice models that do not express VEGF protein in skeletal muscles. Null mice display a 90% and 80% decrease in VEGF protein the gastrocnemius and cardiac muscles respectively. Additionally, these null mice displayed decreases in capillary density in both muscle types and running endurance and speed were greatly reduced, in comparison to wild-type animals engaging in the same exercise pattern (Olfert et al., 2009). Without the peripheral expression of VEGF, animals exhibited intolerance to aerobic exercise, likely due to the lack of vascular development in the muscles. Studies using human participants have found similar effects in the periphery. After engaging in single-leg knee extensions in either a normoxic or hypoxic environment, VEGF mRNA levels were measured in the vastus lateralis muscle. In both environments, VEGF mRNA was significantly increased when compared with measures taken prior to exercise (Richardson et al., 1999). Also, plasma levels of VEGF protein have been found to increase immediately post exercise in human male participants and remain elevated for up to two hours post exercise. No elevations were found in sedentary participants (Kraus, Stallings III, Yeager, & Gavin, 2004).

Exercise that results in VEGF elevations in the periphery produces similar effects in the brain. Tang, Xia, Wagner, and Breen (2010) investigated VEGF transcriptional activation following exercise in the brain, lung, and skeletal muscle. Mice engaged in one treadmill running session for 1 hour, at 24 m/min. Exercise was found to increase VEGF mRNA and protein in the lungs, skeletal muscles, and hippocampus, but not in the heart or liver. This increase in hippocampal VEGF suggests that aerobic exercise patterns that result in VEGF mRNA increases in peripheral regions produce similar effects in the brain (Tang et al., 2010). Ding et al. (2004) had rats engage in treadmill exercise for 30 minutes per day for one, three, or six weeks. One week of exercise produced slight increases in VEGF mRNA, and after three weeks of exercise VEGF mRNA levels robustly increased. Microvessel density increased significantly after three weeks of exercise and this increase remained after exercise was withdrawn; expression of VEGF mRNA decreased after the withdrawal of exercise (Ding et al., 2004). In aged rats, treadmill exercise for 80 minutes per day, for three weeks, significantly increased cerebral microvessel density, which was associated with elevations in VEGF mRNA and protein (Ding et al., 2006). Exercise also alleviated depression-like behavior in rodents through VEGF regulated angiogenesis. Exposure to chronic stress led to depressive-like symptoms in animals, along with decreases in hippocampal blood vessel density and neurogenesis. However, when animals were then exposed to regular exercise training, there were improvements in their depression-like behavior and increased blood vessel density and neurogenesis. Treatment with SU1498, a VEGF Flk-1 receptor antagonist, blocked these effects (Kiuchi, Lee, & Mikami, 2012).

VEGF Receptors. VEGF would not exert these angiogenic effects without signaling through its two high-affinity receptors: FMS-like tyrosine kinase receptor (Flt-1, also termed VEGFR1) and fetal liver kinase 1 receptor (Flk-1, also termed KDR or VEGFR2). Both

receptors have a single transmembrane region, seven immunoglobulin-like domains in the extracellular domain, and its consensus tyrosine kinase sequence contains a kinase-insert domain (Ferrara et al., 2003). VEGF also interacts with lower affinity neuropilins, another family of coreceptors (Ferrara et al., 2003). Hypoxia regulates the expression of Flt-1 and Flk-1 (Ferrara & Davis-Smith, 1997). For example following an MCA occlusion in addition to elevations in VEGF expression, both high affinity receptors were also upregulated in the ischemic border after 48 hours reperfusion and later in the ischemic core (Marti et al., 2000).

Flt-1 was the first of the two high-affinity VEGF receptors identified (Ferrara et al., 2003). Like VEGF itself, Flt-1 expression is increased by hypoxia, likely through HIF-1α dependent signaling (Ferrara et al., 2003). Flt-1 null mice died *in utero* after 8.5- 9.5 days. Endothelial cells differentiated normally; however, assembly of these cells was abnormal. They were disorganized and did not form normal vascular channels (Fong, Rossant, Gertsenstein, & Breitman, 1995). In addition to regulating the initial organization of vasculature during development, Flt-1 also may remain active after new vessels are finished growing, maintaining the organization of the vasculature (Ferrara & Davis-Smyth, 1997). Flt-1 may serve as a negative regulator of the angiogenic actions of VEGF. Its tyrosine kinase activity is weaker than Flk-1, and it can serve as a trap for VEGF, preventing vascular overgrowth (Shibuya, 2006).

While Flt-1 serves as a regulator of VEGF activities and organizes the vasculature, Flk-1 is responsible for the mitogenic effects of VEGF and has a leading role in the development of endothelial cells (Millauer et al., 1993). VEGF binds to Flk-1, which has strong tyrosine kinase activity and a major role in angiogenic signaling. Flk-1 null mice were generated and found to die *in utero* after 8.5-9.5 days, due to defects in endothelial cell development. There were no blood islands detected by 7.5 days of development, no observations of organized blood vessels,

and haematopoietic progenitors were significantly reduced. This suggests Flk-1 is critical for blood island formation and vasculogenesis (Shalaby et al., 1995; Millauer et al., 1993). In sum, these two high affinity receptors serve slightly different function; however, both are critical for the proper formation of a new vessel. Deletion of Flk-1 and Flt-1 are both lethal during embryogenesis. However, the reason for this lethality differs. Flk-1 required for the growth of new vessels and Flt-1 is required for the organization of these newly birthed vessels (Shibuya, 2006).

Like VEGF, Flk-1 and Flt-1 are elevated in both the brain and periphery in response to aerobic exercise. In the periphery, Lloyd et al. (2003) found significant increases in Flk-1 and Flt-1 in the skeletal muscles of rats after completing one day of treadmill exercise training. Expression of both receptors declined to near baseline levels after nine days of training. It is interesting to note that both receptors increased and decreased at similar times. Furthermore, these elevations in receptor levels occurred in correlation with increases in VEGF mRNA and occurred prior to significant increases in capillary density (Lloyd et al., 2003). Others have found significant elevations in VEGF mRNA and Flk-1 expression in the mouse skeletal muscle as early as six hours after a one-hour bout of treadmill of exercise (Kivela et al., 2008). In human skeletal muscle, both Flk-1 and Flt-1 mRNA were elevated four hours after a one-hour bout of cycle ergometer (stationary bicycle) exercise. VEGF mRNA was elevated at two hours, slightly earlier than the receptors, but remained elevated at four hours post exercise (Galvin et al., 2004).

In the brain, Flk-1 and Flt-1 receptor elevations have been detected in the cerebellum and motor cortex following exercise (Bulinski, Thompson, Powell, & Swain, 1999; Bulinski, Thompson, Powell, Sikorski, & Swain, 2000). Rats engaged in voluntary wheel running for 0, 2, 4, 10, or 30 days, and after just two days Flk-1 expression was significantly elevated in the

cerebellar paramedian lobule. At this two-day timepoint, Flt-1 was significantly decreased (Bulinski et al., 1999). This difference is likely due to the differing roles of the two receptors. Flk-1 is required to induce the growth of a new vessel, and Flt-1 is important later to organize the newly formed capillaries into the preexisting vasculature (Swain et al., 2012). In the motor cortex, Flk-1 was significantly elevated after 10 days of voluntary wheel running, which is delayed in comparison the cerebellum. However, this increase in Flk-1 labeling coincided temporally with the onset of angiogenesis (Bulinski et al., 2000). In the hippocampus, low intensity treadmill running for one week produced elevations in Flk-1 mRNA (Lou et al., 2008).

VEGF and Neurogenesis. Some evidence suggests VEGF has a direct role in regulating neurogenesis, in addition to its role in angiogenesis. In a seminal paper, Cao et al. (2004) found that VEGF mediates hippocampal neurogenesis. VEGF was overexpressed in mice, by way of gene transfer, and this overexpression resulted in a two-fold increase in environmental enrichment associated hippocampal neurogenesis, and this increase correlated with improved performance on the MWM. Additionally, inhibiting expression of VEGF completely prevented neurogenesis induced by environmental enrichment (Cao et al., 2004). Few studies have directly investigated the role of VEGF in exercise-induced neurogenesis, but some evidence exists. One week of treadmill exercise enhanced neurogenesis in the dentate gyrus and this increase corresponded with increased Flk-1 mRNA expression, in addition to other growth factors important for neurogenesis such as BDNF (Lou et al., 2008). Furthermore a peripheral blockade of VEGF completely abolished running-induced hippocampal neurogenesis (Fabel et al., 2003). Fabel et al. (2003) suggest VEGF is a critical somatic regulator of hippocampal neurogenesis and that VEGF itself may signal independently of the key signaling pathways typically suggested to be involved in neurogenesis.

Regulation of VEGF. Another interesting aspect of the Fabel et al. (2003) experiment was the blockade of peripheral VEGF. Many studies suggest local transcription of VEGF, regulated by HIF-1α, is critical in regulating neurovascular changes that occur in response to aerobic exercise (Berggren, 2013). However, this study highlights the prominent role of peripheral VEGF in hippocampal plasticity. A peripheral VEGF blockade abolished running induced neurogenesis but had no detectable effects on baseline neurogenesis in inactive controls. Peripheral VEGF does not seem to be a component of regulating neurogenesis under basal conditions; however, it is important for exercise-induced changes (Fabel et al., 2003).

In addition to the contributions of peripheral VEGF in signaling neurovascular changes in the brain, HIF-1α seems to be the key regulator of VEGF expression. However, HIF-1α is not the only regulator of VEGF expression. Even when HIF-1 activity was blocked, animals exhibited some response to hypoxia. Furthermore, animals were not VEGF deficient in comparison to wild-type animals (Kotch et al., 1999). This suggests there are other mechanisms, independent of HIF-1α, mediating VEGF expression.

Another transcription factor involved in VEGF regulation, independent of HIF-1α, is peroxisome proliferator-activated receptor cofactor-1α (PGC-1α). PGC-1α and HIF-1α can regulate VEGF transcription independently; however, they are somewhat coupled in that they are both regulated by intracellular oxygen availability (Ohno, 2012). PGC-1α is a critical regulator of mitochondrial biogenesis and mediates signaling in response to oxygen and glucose deprivation. Unlike HIF-1α, PGC-1α is not critical during embryonic vascularization; PGC-1α null mice are viable (Arany et al., 2008). In adulthood, PGC-1α has critical roles in the angiogenic response to ischemia (Ohno, 2012). PGC-1α is a strong regulator of VEGF expression in cultured skeletal and muscle cells *in vivo* (Arany et al., 2008). These null mice

show failures to reinstate blood flow to limbs after ischemic insult, and overexpression of PGC-1α in skeletal muscles protects against the effects of ischemia (Arany et al., 2008). PGC-1α is critical for exercise-induced increases in skeletal muscle VEGF. PGC-1α knockout and wild-type mice went through five weeks of exercise training, which involved treadmill running five times per week at 14 m/min for one hour *and* access to an exercise wheel in home cages. Wild-type mice showed approximately a 60% increase in VEGF mRNA and protein in comparison to inactive control mice in the quadriceps muscles. There was no change in VEGF protein in the PGC-1α knockout mice post training. Furthermore, VEGF protein levels were 60-80% less in the knockout mice compared to wild-type animals before and after the exercise training began. This finding suggests PGC-1α has a critical role in regulating VEGF protein during basal and exercise conditions in skeletal muscles (Leick et al., 2009). No studies have investigated the effects of PGC-1α in the brain in response to exercise induced angiogenesis; however it has been found to be important in regulating angiogenesis in response to cerebral ischemia (Chen et al., 2011).

Exercise Pattern and Intensity

Most studies suggest that aerobic exercise promotes robust neurovascular changes in the brain. However, the methods of exercise used across these studies vastly differ in pattern and intensity. Numerous studies indicate exercise promotes plasticity in the neurovascular system and provides behavioral benefits, including improved learning and memory task performance and recovery of motor function following cerebral ischemia (Ra et al., 2002; Leasure & Jones, 2008; Ke, Yip, Li, Zhen, & Tong, 2011). However, the pattern and intensity of physical exercise required to produce the most robust effects has not been well established.

There are two exercise patterns most used within the literature: voluntary or forced. Voluntary exercise usually involves wheel running, which allows the rodent unrestricted access

to a running wheel, typically located in the home cage. In this condition, animals control the exercise and it generally involves running at rapid speeds for short durations (Leasure & Jones, 2008). The other is forced exercise, and this pattern generally involves the animal being removed from the home cage and placed on a treadmill to run. There is usually a shock-grid to prevent the animal from stopping. In this condition, animals usually run for a set duration, at slower speeds with no breaks (Leasure & Jones, 2008). Another forced pattern, used less often, is forced swim exercise. In forced swim, the animal is placed in the pool with no platform to escape. Like voluntary wheel running and forced treadmill running, forced swim also produces similar neurovascular benefits (Ra et al., 2002). Although both patterns exert neurovascular changes to some degree, findings differ when directly compared.

Ke and colleagues (2011) compared the effects of voluntary, involuntary, and forced exercise on BDNF expression and motor function recovery following cerebral ischemia. All animals underwent a MCA occlusion and were divided into four groups: inactive control, voluntary wheel running, forced treadmill exercise, and exercise that involved two implanted electrodes placed on the hind limb muscles on the side of the body affected by the stroke. This stimulation mimicked the typical rodent gait. Forced exercise animals ran at 20 m/min, for 30 minutes daily and the voluntary exercise group had free access to running wheels in the home cages. All animals completed their respective exercise regimens for seven days after the transient MCA occlusion. Voluntary wheel running produced significantly higher BDNF expression in the hippocampus, in comparison to the forced exercise group and inactive controls. Furthermore, the voluntary exercise group performed significantly better on the motor recovery test, compared to the other groups. Serum corticosterone was elevated in the forced exercise animal. It is well established that corticosterone has detrimental effects on both angiogenesis and neurogenesis in

the hippocampus (Mirescu & Gould, 2006). Ke et al. (2011) suggest these high corticosterone levels downregulated BDNF expression in the forced exercise animals. In this experiment, voluntary exercise produced the most robust expression of BDNF and motor recovery following cerebral ischemia (Ke et al., 2011).

In contrast, Hayes et al. (2008) found that forced exercise was neuroprotective against ischemic stroke. Voluntary exercise was not protective. This study had five groups: inactive control, forced treadmill exercise, voluntary wheel running, restraint stress, and electric shock. The forced exercise group ran at a speed of 30 m/min for 30 minutes per day, five days per week, and voluntary wheel runners had free access to wheels in their home cages seven days per week. Animals in the restraint stress group were placed in a restrainer for 120 minutes seven days per week, and the shock group received 15 shocks, at variable times, over the course of 30 minutes seven days per week. Animals in the shock group were exposed to the same shock grid used in the forced treadmill condition to keep the animals running, and they were shocked a comparable amount of times. These two stress conditions were to assess the effects of corticosterone alone on neuroprotection. All animals engaged in their respective regimens for three weeks, and then a stroke was induced via a two hour MCA occlusion. All animals were sacrificed 48 hours after the occlusion. Interestingly, the percentage of brain infarct volume was significantly lower in forced exercise group, compared to all other conditions. Additionally, researchers found no correlation between the distance run in the voluntary exercise group and the degree of neuroprotection. On average, the voluntary exercise animals ran 4,828 meters per day, in comparison to the 900 meters run per day by the forced exercise animals. Thus running more was not more neuroprotective. Hayes and colleagues (2008) conclude that forced exercise, with the stressful component of a shock grid, is most neuroprotective against cerebral ischemia. When

shock or restraint stress was presented alone, with no exercise, they were not neuroprotective. Hayes et al. (2008) suggest the combination of stress and forced exercise significantly reduced brain damage.

Ke et al. (2011) found that voluntary exercise produced the most robust motor recovery and suggested corticosterone elevations produced by forced exercise negatively affected BDNF expression and limited recovery. Hayes et al. (2008) suggested that the combination of corticosterone and forced treadmill exercise was neuroprotective. Although Ke et al. (2011) investigated the effects of different patterns of exercise post cerebral ischemia, and Hayes et al. (2008) analyzed the neuroprotective effects of different patterns prior to ischemia, these findings are profoundly different.

In another study investigating the effects of voluntary and forced exercise in cerebral ischemia recovery, Ploughman et al. (2007) had animals exercise for 60 minutes in either a voluntary or forced condition, two weeks after cerebral ischemia. Forced exercise required animals to run on a motorized running wheel at 4 m/min for five minutes and 9 m/min for 20 minutes. The voluntary exercise group began by running for five minutes and then progressed to running for 60 minutes. Exercise took place for seven days. Animals were sacrificed 0, 30, 60, and 120 minutes post exercise. In the ischemic hemisphere, BDNF was upregulated for up to two hours following voluntary running. BDNF was only elevated 20 minutes following forced motorized wheel running. Their findings suggested that forced exercise induced a more rapid, short-term increase in BDNF compared to voluntary running (Ploughman et al., 2007). In a previous study, Ploughman (2005) also had animals exercise following focal cerebral ischemia. The motorized forced exercise condition for 30 or 60 minutes was more effective than voluntary running in the elevation of BDNF and synapsin-1 in the sensorimotor region and hippocampus in

the intact hemisphere, immediately following exercise. Ploughman et al. (2007) sheds light on this effect. Forced motorized wheel running produces a more rapid increase in these growth factors, which can be detected immediately post exercise.

Instead of investigating the effects of different exercise patterns on cerebral ischemia, the same research group assessed the differences in cerebral metabolism following forced or voluntary exercise (Kinni et al., 2011). Animals engaged in either voluntary wheel running or forced treadmill running, following the same exercise procedures described in their previous paper (Hayes et al., 2008). After three weeks of exercise, HIF-1α mRNA and protein was significantly elevated in the forced exercise group, compared to the voluntary exercise and control groups. Although expression of HIF-1α mRNA and protein in the voluntary exercise group was significantly lower than the forced condition, it was elevated in comparison to controls. Like in the Hayes et al. (2008) study, voluntary exercise animals completed approximately 4,432.2 meters per day and forced exercise animals completed 900 meters per day. Again, like in the previous study, although voluntary exercise animals ran significantly further, the shorter distance forced exercise group displayed higher levels of HIF-1α. In Hayes et al. (2008), the short distance forced exercise groups displayed the most robust neuroprotective effects. In this study, the authors again suggest that stress from the forced exercise may be what caused the greater HIF-1α elevations in the forced exercise group. However, they also suggest that forced exercise, being more strenuous, may produce more robust metabolic changes. Animals were required to run 900 meters at a rapid speed, with no breaks, and this may have caused greater metabolic deficits than voluntary exercise, where animals could exercise on and off throughout the day (Kinni et al., 2011).

In addition to differences in patterns, differences in intensity may also affect the degree of neurovascular changes that occur following exercise. Lou et al. (2008) compared the effects of different exercise intensities on hippocampal neurogenesis. Additionally, they measured BDNF, VEGF, and Flk-1 mRNA in the hippocampus. Animals exercised at either a low (5 m/min for five minutes, 8 m/min for five minutes, 11 m/min for the remaining 20 minutes) moderate (8 m/min for five minutes, 11 m/min for five minutes, and 14m/min for the remaining 20 minutes) or a high (8 m/min for five minutes, 11 m/min for five minutes and 22 m/min for the remaining 20 minutes) intensity for one week. Low and moderate intensity exercise produced the most robust $BrdU^{+}$ neuronal labeling in the dentate gyrus. Furthermore, gene expression for all molecules measured, including BDNF, VEGF, and Flk-1 mRNA were significantly greater in the low intensity group than the high intensity group. These findings suggest that neurogenesis is affected by exercise intensity and contrast Kinni et al. (2011), which suggested the high exertion associated with forced exercise make it more neuroprotective. Lou et al.'s (2008) results suggest the effects of exercise on neurogenesis is highly dependent on intensity, but in the opposite direction of other studies. Low, rather than high intensity forced exercise produced the most robust effects. Lou et al. (2008) suggest that high intensity exercise may reduce BDNF mRNA expression via its effects on energy metabolism.

Leasure and Jones (2008) conducted a study comparing the differential effects of forced and voluntary exercise on neurogenesis and behavior. Rats ran for five days per week, for eight weeks either voluntarily, using a wheel attached to their cages, or forced, using a motorized running wheel. Forced exercise animals matched the distances voluntary exercise animals ran per day. Voluntary wheel runners began running near 1,000 meters and gradually increased to 2,300 meters by eight weeks. The forced exercise group followed the same pattern. The forced exercise

groups also ran in a manner that mimicked that of the voluntary exercise group: short bursts of exercise with frequent breaks. The speed of the motorized wheel varied, and brief breaks were randomly included. After eight weeks of exercise, there were no differences between the groups on MWM performance. Both exercise groups displayed increased percentages of surviving neuronal progenitor cells, but the pool was significantly larger in the forced exercise group. Corticosterone levels were measured and there were no differences between the groups. Researchers suggested that after eight weeks of exercise the animals likely adapted to any effects of stress. Instead, they suggest that differences in exertion may lead to the differential effects of forced and voluntary exercise. Voluntary exercise groups ran at higher speeds for shorter durations, and forced exercisers ran at slower speeds for longer durations. This difference may have contributed to these findings. Furthermore, this suggestion is similar to that of Kinni et al. (2011), which suggested that the forced exercise required more exertion.

Few studies have investigated differences in exercise intensity within the voluntary exercise paradigm. However, unlimited access to voluntary wheels produces enhanced performance on the MWM, but not restricted access (van Praag et al., 1999; van Praag et al., 2007). van Praag et al. (2007) allowed mice to run on voluntary exercise wheels for two hours per day, for six weeks. After six weeks of exercise, MWM behavioral testing began. Mice completed two trials per day for eight days, and probe trials were completed on day eight, four hours after the last training session and again 24 hours later. Interestingly, mice that engaged in voluntary exercise did not perform significantly better than controls on the MWM. In previous studies, using this same paradigm, mice with unlimited access to voluntary wheel running performed significantly better than inactive controls (van Praag et al., 1999). These findings suggest that distance run affects cognition, and likely the degree of underlying neurovascular

changes. This contrasts with previous studies that propose that distance run does not affect the degree of neurovascular change that takes place following aerobic exercise (Hayes et al., 2008; Kinni et al., 2008).

Purpose

Most studies indicate that aerobic exercise beneficially affects angiogenesis, neurogenesis, and cognition. However, many of these studies use only one pattern and intensity of exercise. Within the studies that do directly compare exercise pattern and/or intensity, findings still vary (Kinni et al., 2008; Ke et al., 2011; Ploughman et al., 2007). Furthermore, most studies investigating the differential effects of exercise pattern and/or intensity focus on neuronal, rather than vascular changes, and few studies have investigated differences in intensity using a voluntary exercise paradigm, most use forced exercise (Lou et al., 2008). An additional gap in the literature includes investigating the differential effects of voluntary and forced exercise on the *acute* changes that occur in association with angiogenesis. Previous experiments had animals run for longer durations, ranging from 1-8 weeks, before quantifying neurovascular changes (Kinni et al., 2008; Lou et al., 2008; Leasure & Jones, 2008). However, these angiogenic changes begin within hours of exercise onset (Kerr & Swain, 2011).

This study aimed to understand how exercise intensity and pattern affects the expression of Flk-1 and Flt-1 receptors in the hippocampus and cerebellum after an acute bout of exercise. It was hypothesized that VX-U would display the highest expression of Flk-1 and Flt-1 receptors, followed by VX-H, VX-L, and FX-L. Animals in the FX-H group were expected to have the lowest expression of Flk-1 and Flt-1 receptors in both the hippocampus and cerebellum.

Method

Subjects

Seventy-four male, Long Evans hooded rats (175-200 grams) were purchased from Envigo (Madison, WI). Animals were divided into eight groups: Voluntary Exercise-Unrestricted (VX-U; n=10), Voluntary Exercise- High Intensity (VX-H; n=10), Voluntary Exercise- Low Intensity (VX-L; n=10), Forced Exercise- High Intensity (FX-H; n=10), Forced Exercise- Low Intensity (FX-L; n=10), Inactive Control- Unrestricted (IC-U; n=8), Inactive Control- High Intensity (IC-L; n=8), and Inactive Control- Low Intensity (IC-H; n=8). All animals were housed individually in standard shoebox cages (47.82 x 20.32 x 22.86 cm^3) for a one-week acclimation period. After the one-week acclimation, the VX-U group were housed in cages with attached running wheels. Animals were housed on a 12-hour light/dark cycle with access to enviro-dri and food and water *ad libitum* throughout the experiment. The Institutional Animal Care and Use Committee (IACUC) at the University of Wisconsin- Milwaukee approved all procedures.

Materials

All animals, except ICs, engaged in an exercise regimen. Animals in the VX-H and VX-L groups were placed in a cage with an attached running wheel and remained in this cage until 1,000 (high intensity) or 500 (low intensity) revolutions were reached, respectively. Once these revolutions were reached, animals were returned to their home cages. The FX-H and FX-L groups were placed in motorized running wheels at a speed of ~9 m/min (See Figure 1). Animals engaged in forced running until the FX-H group reached 1,000 revolutions and the FX-L group reached 500 revolutions. VX-U was housed in cages with attached wheels, identical to those used by the VX-H and VX-L groups. All exercise wheels had a one-meter circumference, thus

1,000 and 500 revolutions equated to 1,000 and 500 meters, respectively. Although all wheels were of the same diameter, there were two different styles. Half of the wheels were mounted inside the standard shoebox cage and half of the wheels were attached to the back of the standard shoebox cage (see Figures 2 and 3). Exposure to the different wheel styles was counterbalanced within the different voluntary exercise conditions.

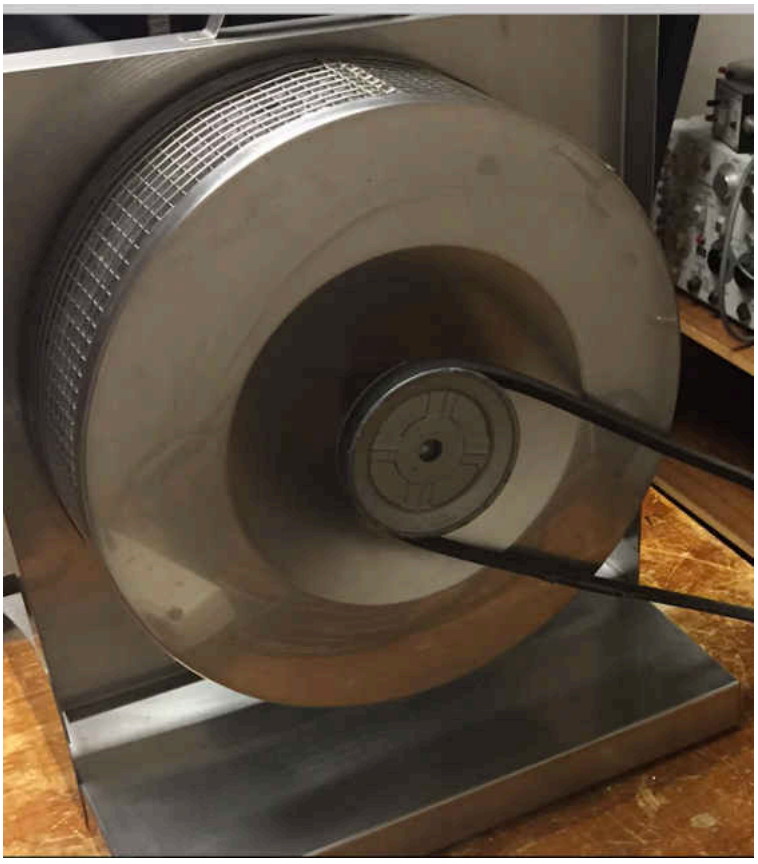

Figure 1: Motorized running wheel for forced exercise conditions. This belt was attached to a motor that allowed the speed to be controlled by the experimenter.

Figure 2: Voluntary exercise cage with running wheel attached in the back.

Figure 3: Voluntary exercise cage with running wheel attached inside the cage.

Procedure

Upon arrival, animals acclimated in their home cages for one week. During acclimation, no groups had access to running wheels. After this first week, all animals were pre-exposed to voluntary wheel running. All animals, including those in forced exercise and inactive control conditions, underwent this voluntary exercise pre-exposure for two days. For 10 minutes daily, all animals were placed individually into cages with voluntary exercise wheels. Animals were allowed free access to the wheels and controlled the duration and speed of exercise. After the 10 minute pre-exposure, wheel revolutions were recorded and animals were returned to their home cages. The cages used for pre-exposure were the same cages used in the voluntary exercise conditions. Animals in the forced condition also received pre-exposure training in effort to familiarize them with wheel running, prior to engaging in forced exercise on the motorized wheel. IC animals engaged in the pre-exposure training to control for the effects of the pre-exposure exercise on vascular changes.

After the two days of pre-exposure, all animals began their exercise conditions, except IC groups, which remained inactive in their home cages for 24 hours. All animals began exercise at 9:00 am. VX-U rats were moved to cages with attached wheels and allowed unrestricted access to the running wheels for the full 24 hours. At the end of the 24-hour period, the number of revolutions completed was recorded. VX-H and VX-L rats were also moved to the same cages with attached running wheels, but they were returned to their home cages after reaching 1,000 revolutions (high intensity) and 500 revolutions (low intensity), respectively. Research assistants periodically monitored the number of revolutions these animals completed and were responsible for removing animals from the exercise cages once the required number of revolutions was reached. FX-H and FX-L animals were required to run on a motorized wheel at ~9 m/min. FX- H

and FX-L rats were returned to their home cages after reaching 1,000 and 500 revolutions, respectively. Research assistants monitored FX-H and FX-L animals for the whole duration of exercise.

Twenty-four hours after the start of exercise, all animals were sacrificed by immersion in a carbon dioxide chamber. Animals were decapitated, and brains were removed and snap frozen in chilled isopentane. Brains were stored at -80 °C until prepared for immunohistochemistry (IHC). One hemisphere per animal was randomly selected for IHC. Brains were hemisected and prepared for sectioning, at 12 μm, using a Leica CM 3050 S cryostat (Wetzlar, Germany). The cerebrum was sliced coronally, through the entire dorsal hippocampus. Sixteen sections per animal were randomly selected, and these sixteen sections were placed on two different slides (eight sections for Flk-1 IHC and eight sections for Flt-1 IHC). The cerebella were sliced sagittally, through the paramedian lobule, and the collection of sections was identical to that of the hippocampi. Additionally, two sections per animal were also collected to serve as negative controls: one section for Flk-1 and one section for Flt-1. These sections underwent the IHC procedure; however, they never received the primary antibody. The purpose of the negative control is to test antibody specificity; there should be no labeling on these negative control sections.

After tissue was sectioned, slides dried overnight. Before beginning IHC, targeting Flk-1 (Santa Cruz Biotechnology, Dallas, TX) and Flt-1 (Santa Cruz Biotechnology, Dallas, TX), tissue was fixed by immersion in chilled acetone (-20 °C) for 10 minutes. Tissue was then removed and allowed to dry for 10 minutes, prior to beginning IHC. After tissue was dry, all slides went through two, five minutes washes in phosphate buffered saline (PBS). Peroxidase activity was then blocked by completion of a 10-minute wash with 0.3% hydrogen peroxide in

PBS. After endogenous peroxidase activity was blocked, two more, five minute PBS washes followed. Slides were then incubated in a humidity chamber for one hour at room temperature, in a blocking solution (prevent non-specific binding) containing 10% goat serum, 0.5% triton X (10% concentration) and PBS. Lastly, the blocking solution was drained off and tissue was incubated overnight, at 4 °C, in the primary antibody solutions (1:500 dilution for both Flk-1 and Flt-1). The primary antibody solution was pipetted onto each slide (50µl per section), and coverslips were placed on top of the primary antibody solution. The weight of the coverslip created surface tension, keeping the primary antibody from draining off the slide during the overnight incubation. On day two, coverslips were removed and the primary antibody solution was drained off the slides. Slides were then washed in with PBS for 10 minutes, three times. After the rinses, slides were incubated in the secondary antibody (Vector Laboratories, Burlingame, CA) solution (10% serum, 1% secondary antibody, and PBS) for 1 hour and 30 minutes in a humidity chamber at room temperature. After the incubation, the secondary antibody solution was drained off of slides, and tissue was again washed for 10 minutes in PBS, three times. Then, the avidin biotin complex (ABC) was pipetted onto all slides and tissue was incubated at room temperature in the humidity chamber for 1 hour. Lastly tissue was rinsed with three final PBS washes, for 10 minutes each, and reacted in 3-3' diaminobenzidene (DAB) for 5-10 minutes. All slides were then washed in PBS five times, for five minutes each rinse, counterstained with Safranin-O and coverslipped.

All tissue was imaged using a light microscope (Olympus, America, Inc., Center Valley, PA, USA) with an attached SPOT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI, USA). Five sections per animal were randomly selected for imaging at a 400x magnification. Images were taken until the complete structure (dorsal hippocampus or paramedian lobule) was

captured. The stage was moved 0.6 mm in the medial/lateral direction and 0.5 mm in the dorsal/ventral direction across the structure to avoid collecting overlapping images.

After tissue sections were imaged, images from each section were randomly selected for unbiased stereology to determine the total area fraction of Flk-1 and Flt-1 labeling. Images were coded using a random number generator, so the experimenter was blind to the animal's exercise condition during quantification. For quantification, the dorsal hippocampus was divided into three regions of interests: CA1, CA2/3, and DG. The paramedian lobule was divided into two regions of interest: the molecular layer and the granular/Purkinje layers. At least five images per section, per animal, were randomly selected for unbiased stereology. For each image, a 192-point grid was overlaid (see Figure 4). If the point fell on labeling, it was counted. For each region of interest, for each animal, a minimum of 100 points that fall on labeling was counted. Area fractions were calculated for each region of interest by dividing the total number of points that fall on labeling by the total number of points on the grid.

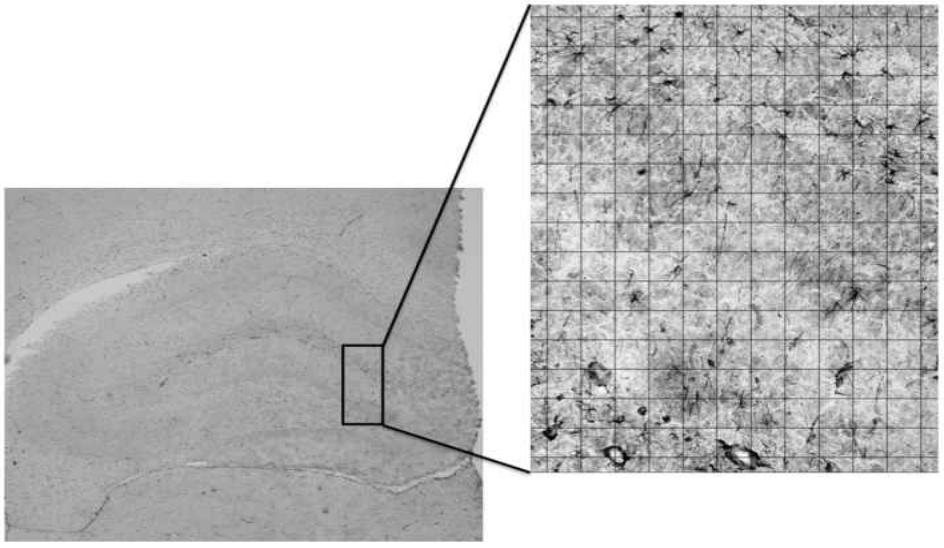

Figure 4: Images of the entire dorsal hippocampus were collected using a 400x magnification and a 192-point grid was overlaid for unbiased stereological quantification.

For each region of interest, a two-way analysis of variance (ANOVA) was completed with pattern (forced, voluntary, or control) and intensity (high, low, or unrestricted) as independent variables and either Flt-1 or Flk-1 area fraction as the dependent variable. Post hoc tests using the least significant difference (LSD) correction were completed to identify specific groups that were different. It was hypothesized that VX-U would display the highest expression of Flk-1 and Flt-1 receptors, followed by VX-H, VX-L, and FX-L. Animals in the FX-H group were expected to have lowest expression of Flk-1 and Flt-1. It was hypothesized that the most robust effects would be found in the CA1 region of the hippocampus and the molecular layer of the paramedian lobule.

Results

Exercise Behavior

Three of the 74 animals in the study did not fully complete their required exercise regimens. Two FX-H animals only completed 900/1,000 revolutions due to fatigue and refusal to run near the end of the session, and one VX-L animal only completed 105/500 revolutions in the 24-hour exercise session. For these animals, area fractions for Flk-1 and Flt-1 labeling were not considered outliers, and thus these animals were not removed from later analyses.

VX-U animals ran an average of 2,348 revolutions within the 24-hour exercise session. The average times to complete the required number of wheel revolutions for VX-L, VX-H, FX-L, and FX-H were also calculated. VX-L required an average of 6.98 hours to complete 500 revolutions and VX-H an average of 11.02 hours to complete 1,000 revolutions. The VX-L animal that failed to complete 500 revolutions was removed from this calculation. FX-L required an average of 0.85 hours to complete 500 revolutions and FX-H an average of 1.79 hours to

complete 1,000 revolutions. Animals in the forced exercise groups ran at a speed of ~9.25 m/min.

Weight changes after completing the 24-hour exercise session were also calculated. IC animals gained an average of 5.33 grams over this period of time. On average, all animals that engaged in exercise lost weight following the exercise session. VX-U lost the most weight: 4.5 grams, followed by FX-L: 2.9 grams, FX-H: 1.9 grams, VX-L: 1.4 grams, and VX-H: 0.2 grams.

Flk-1 and Flt-1 Expression: Hippocampus

Tissue samples from all 74 animals were used in hippocampal analyses for Flk-1 and Flt-1 area fractions. For both Flk-1 and Flt-1, analyses were completed for each region of interest (CA1, CA2/3, and DG), and for the hippocampus as a whole. Each analysis involved running a two-way ANOVA with pattern (forced, voluntary, or inactive control) and intensity (high, low, or unrestricted) as independent variables and either Flk-1 or Flt-1 area fractions as dependent variables.

Flk-1. For the whole hippocampus, a two-way ANOVA indicated a significant main effect for pattern $F_{(2,66)} = 4.965$, $p = 0.010$; see Figure 5. Post hoc tests (LSD) revealed a significantly greater expression of Flk-1 in voluntary exercise groups ($M = 0.185$, $SEM = 0.006$) compared to forced exercise groups ($M = 0.157$, $SEM = 0.007$; $p = 0.001$), and a significantly greater expression in voluntary exercise groups ($M = 0.185$, $SEM = 0.006$) compared to inactive control groups ($M = 0.167$, $SEM = 0.005$; $p = 0.022$). There was no significant difference in Flk-1 expression when forced exercise groups ($M = 0.157$, $SEM = 0.007$) were compared with inactive control groups ($M = 0.167$, $SEM = 0.005$; $p = 0.253$). There was no significant main effect for intensity $F_{(2,66)} = 2.549$, $p = 0.087$; see Figure 6, and there was no significant interaction between pattern and intensity $F_{(3,66)} = 1.357$, $p = 0.264$; see Figure 7.

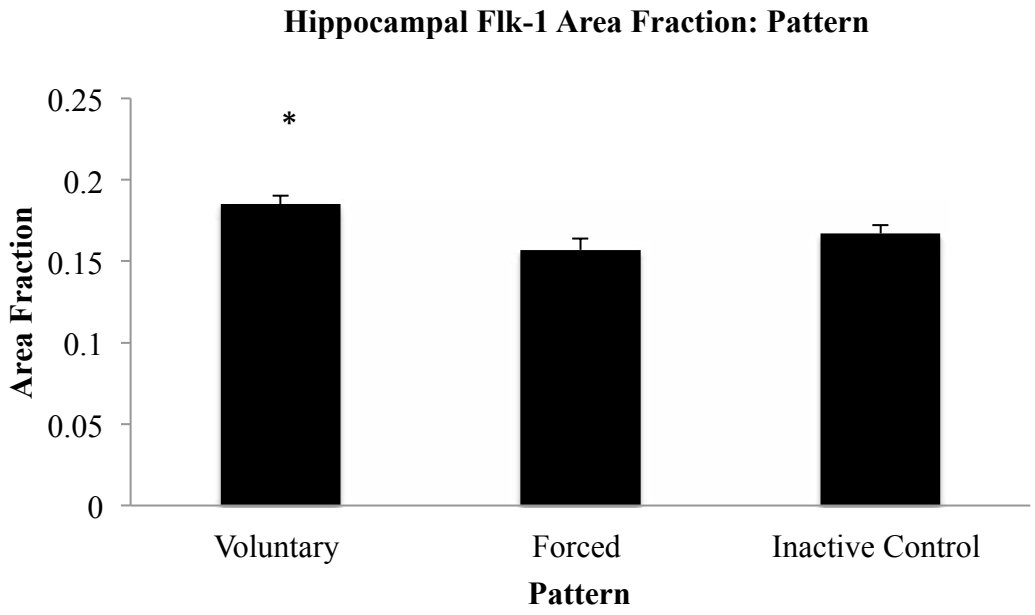

Figure 5: Hippocampal Flk-1 expression based on pattern. Voluntary exercise* had a significantly greater Flk-1 area fraction compared to both forced ($p = 0.001$) and inactive control ($p = 0.022$).

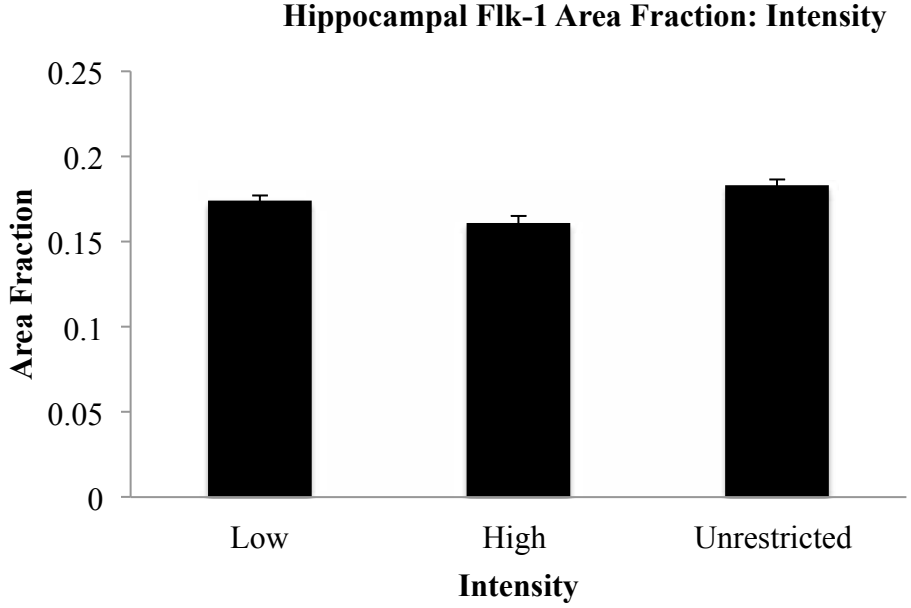

Figure 6: Hippocampal Flk-1 expression based on intensity. There were no significant differences in Flk-1 area fraction between intensities ($p = 0.087$).

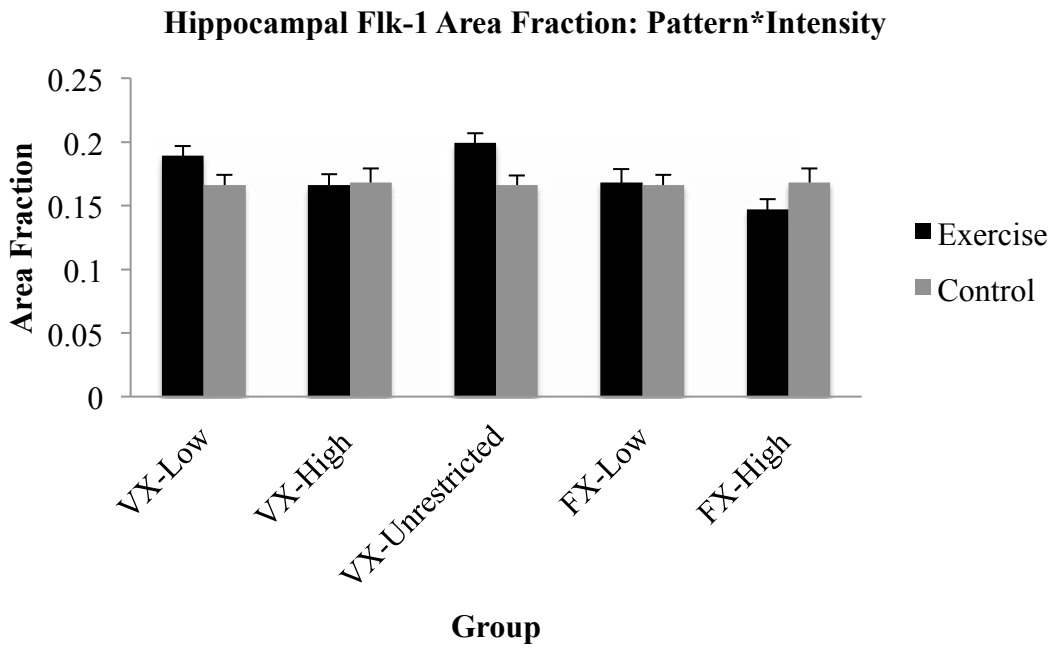

Figure 7. Hippocampal Flk-1 expression based on pattern and intensity. There was no significant interaction between exercise intensity and pattern ($p = 0.264$).

Flk-1 expression in each region of the hippocampus resembled findings in the whole hippocampus. For CA1, a two-way ANOVA indicated a significant main effect for pattern $F_{(2,66)} = 5.017$, $p = 0.009$; see Figure 8. Post hoc tests (LSD) found a significantly greater expression of Flk-1 in voluntary exercise groups ($M = 0.191$, $SEM = 0.006$) compared to both forced exercise groups ($M = 0.161$, $SEM = 0.008$; $p = 0.002$) and inactive control groups ($M = 0.170$ $SEM = 0.006$; $p = 0.015$). Flk-1 expression in forced exercise groups ($M = 0.161$, $SEM = 0.008$) compared to inactive control groups ($M = 0.170$, $SEM = 0.006$) was not significantly different, $p = 0.389$. Like in the whole hippocampus, there was no significant main effect for intensity $F_{(2,66)} = 1.659$, $p = 0.198$; see Figure 9, and no significant interaction between pattern and intensity $F_{(3,66)} = 0.661$, $p = 0.579$; see Figure 10. Findings for CA2/3 and DG were similar to those of CA1, in which there were significant main effects for exercise pattern (see Table 1).

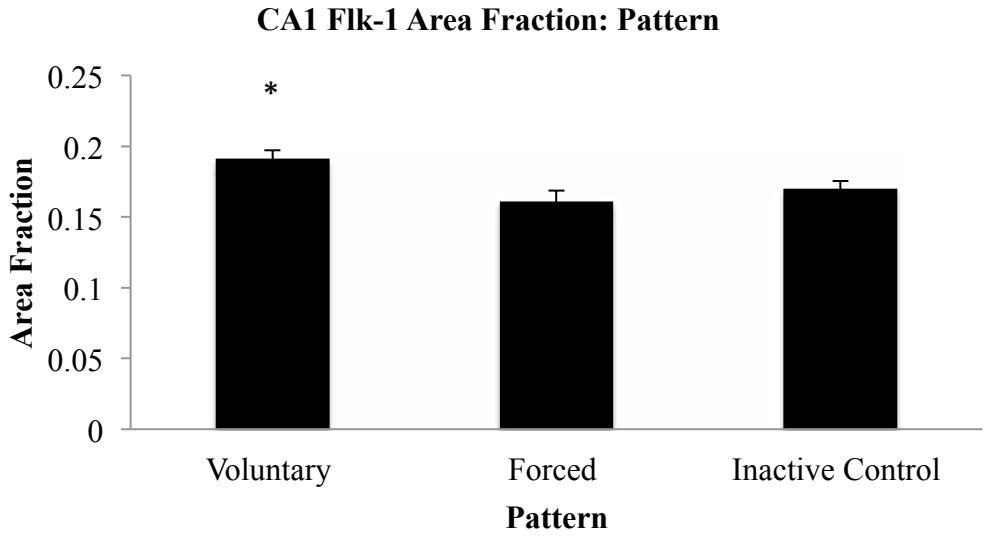

Figure 8: Flk-1 expression based on pattern in CA1. Voluntary exercise* had a significantly greater Flk-1 area fraction compared to both forced (p= 0.002) and inactive control (p = 0.015).

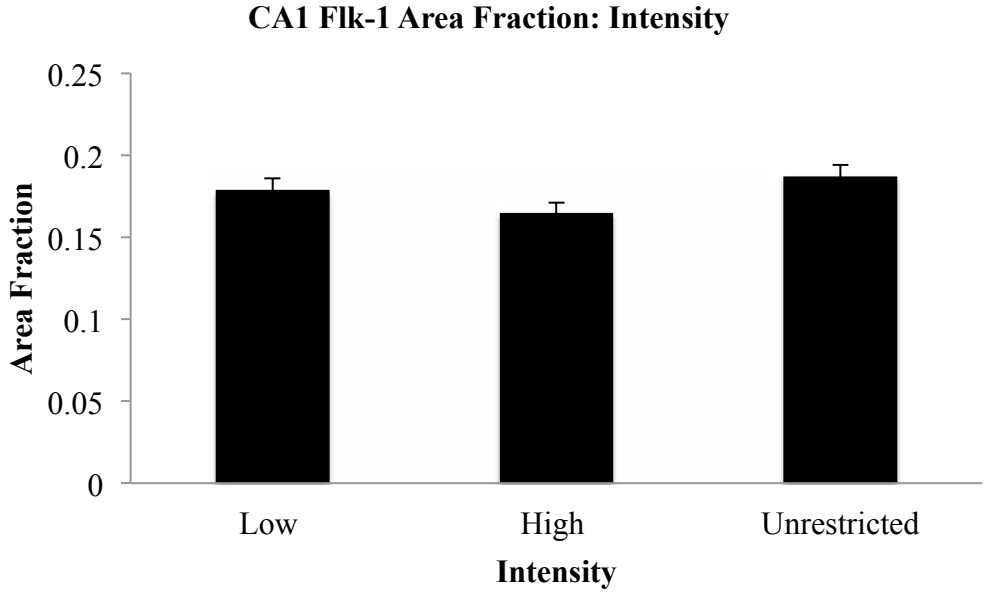

Figure 9: Flk-1 expression based on intensity in CA1. There were no significant differences in Flk-1 area fractions based on intensity (p = 0.198).

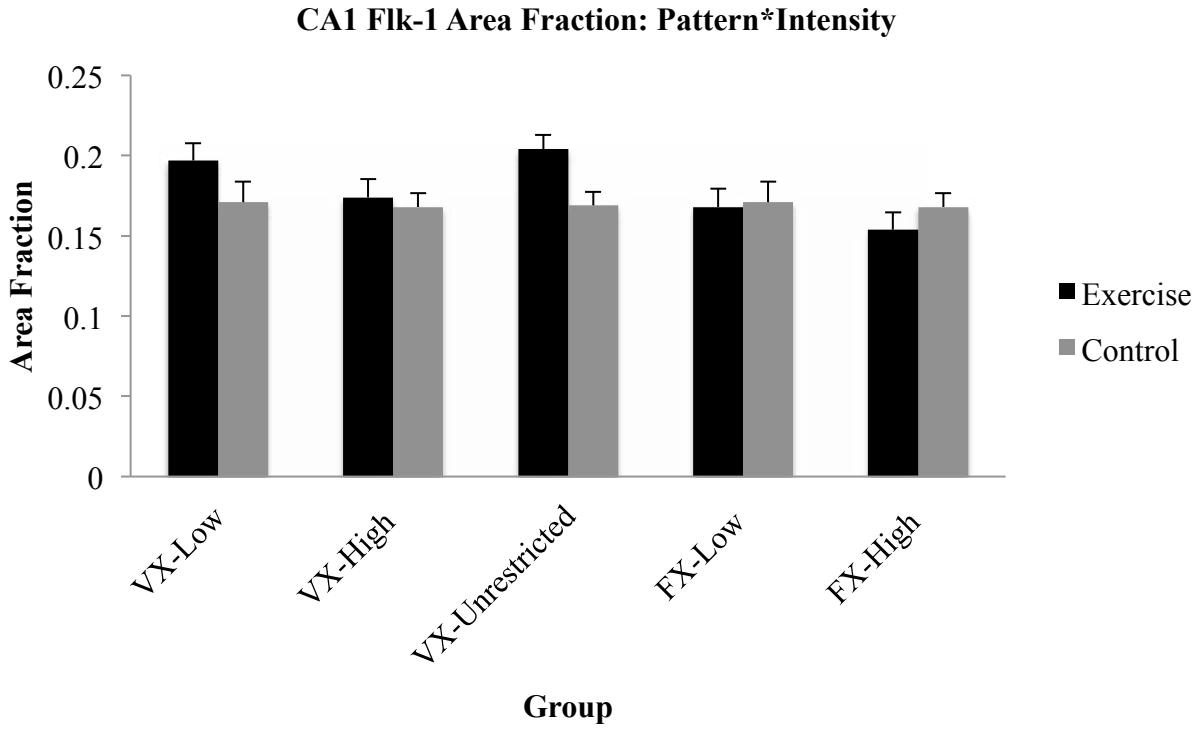

Figure 10: Flk-1 expression based on pattern and intensity in CA1. There was no significant interaction between exercise pattern and intensity ($p = 0.579$).

Table 1

Mean (SEM) and Statistical Significance for Flk-1 Area Fractions: Pattern

	Hippocampal Region		
Pattern	CA1	CA2/3	DG
Voluntary	0.191 (0.006)*	0.176 (0.006)*	0.191 (0.006)*
Forced	0.161 (0.008)	0.151 (0.006)	0.163 (0.007)
Inactive Control	0.170 (0.006)	0.159 (0.006)	0.176 (0.005)
Sig. *(p)*	0.009	0.036	0.031

Flt-1. In the hippocampus, Flt-1 results mirrored those of Flk-1. For the whole hippocampus, a two-way ANOVA indicated a significant main effect for pattern $F_{(2,66)} = 7.362$, $p = 0.001$; see Figure 11. Post hoc tests (LSD) indicated a significantly greater expression of Flt-1 in voluntary exercise groups ($M = 0.110$, $SEM = 0.002$) compared to forced exercise groups ($M = 0.097$, $SEM = 0.003$; $p = 0.003$) and between voluntary exercise groups ($M = 0.110$, $SEM = 0.003$) and inactive control groups ($M = 0.096$, $SEM = 0.003$; $p = 0.001$). There was no significant difference between forced exercise ($M = 0.097$, $SEM = 0.003$) and inactive control ($M = 0.096$, $SEM = 0.003$; $p = 0.915$). There was no significant main effect for intensity $F_{(2,66)} = 0.515$, $p = 0.599$; see Figure 12, and no significant interaction between pattern and intensity $F_{(3,66)} = 0.570$, $p = 0.637$; see Figure 13.

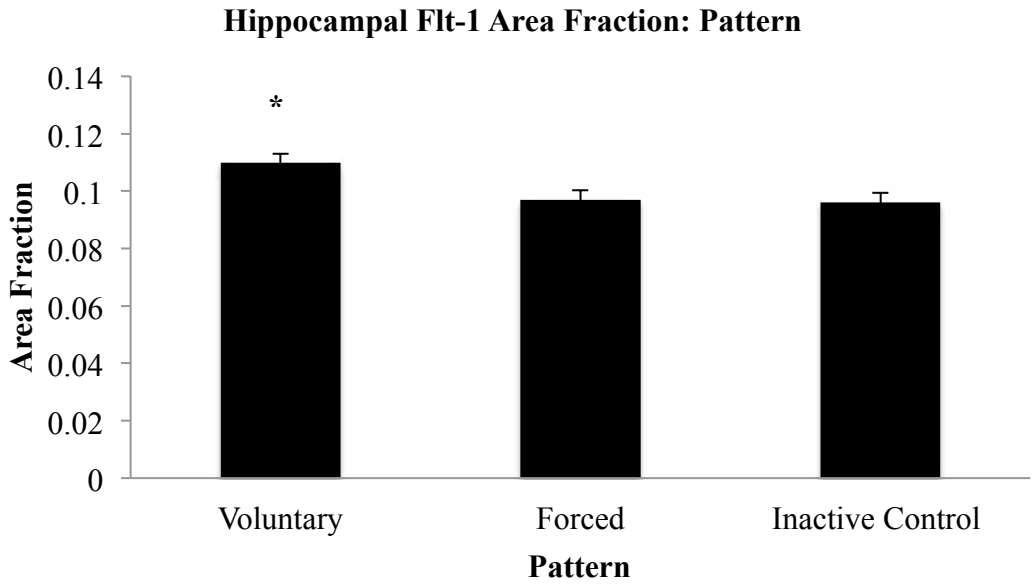

Figure 11: Hippocampal Flt-1 expression based on pattern. Voluntary exercise* had a significantly greater Flt-1 area fraction compared to both forced exercise ($p = 0.003$) and inactive controls ($p = 0.001$).

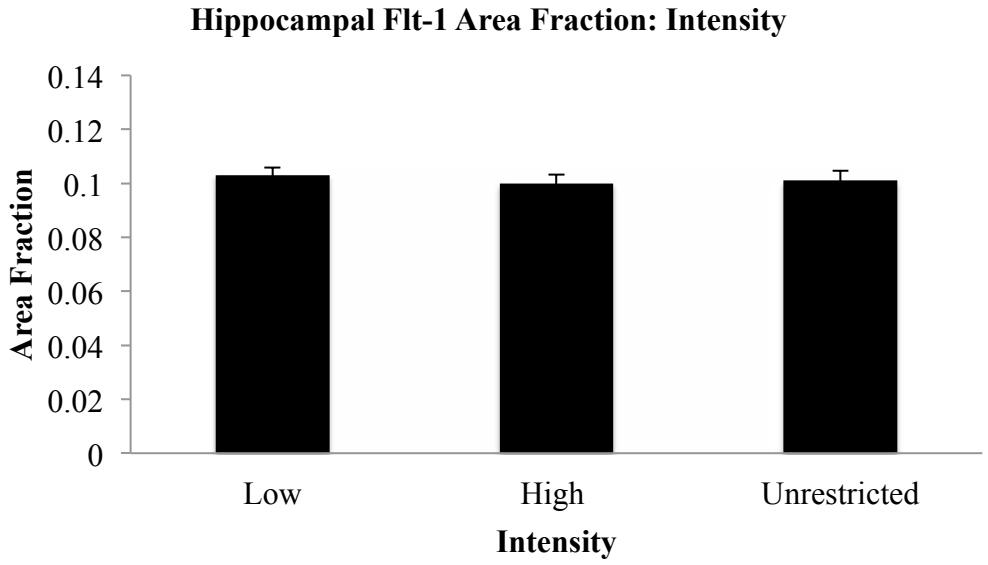

Figure 12: Hippocampal Flt-1 expression based on intensity. There were no significant differences between intensities ($p = 0.599$).

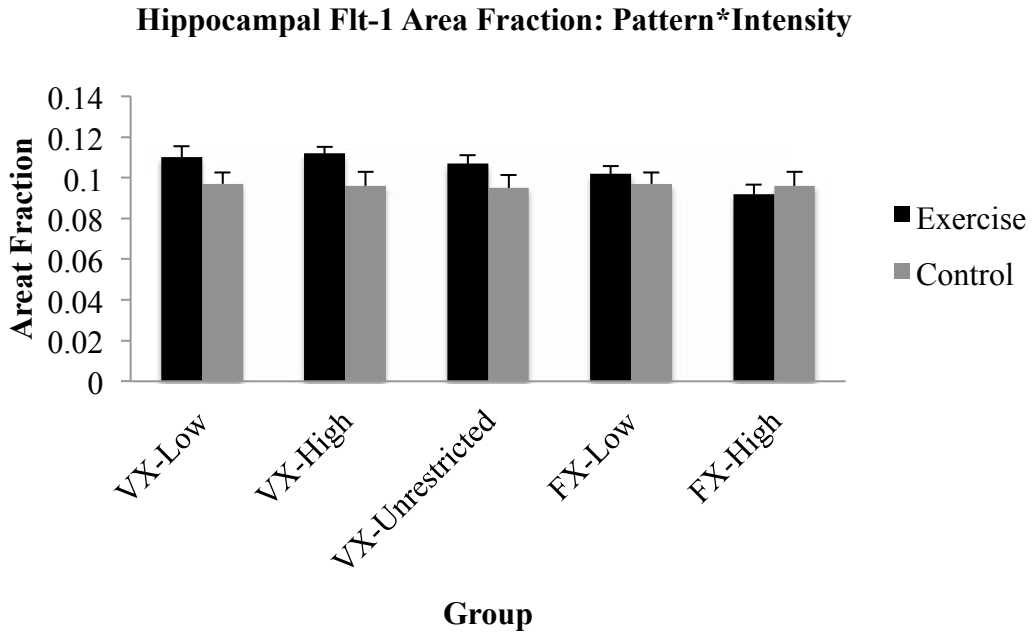

Figure 13: Hippocampal Flt-1 expression based on pattern and intensity. There was no significant interaction between exercise intensity and pattern ($p = 0.637$).

Each region of the hippocampus followed a similar pattern of Flt-1 expression as to that of the whole hippocampus. For CA1, a two-way ANOVA revealed a significant main effect for pattern $F_{(2,66)} = 10.786$, $p = 0.00009$; see Figure 14. Post hoc tests (LSD) indicate Flt-1 expression was higher in voluntary exercise groups ($M = 0.111$, $SEM = 0.003$) compared to forced exercise groups ($M = 0.096$, $SEM = 0.003$; $p = 0.001$) and inactive control groups ($M = 0.093$, $SEM = 0.005$; $p = 0.0003$). There was no significant difference between forced exercise groups ($M = 0.096$, $SEM = 0.003$) and inactive control groups ($M = 0.093$, $SEM = 0.005$; $p = 0.45$). There was no significant main effect for intensity $F_{(2,66)} = 0.376$, $p = 0.688$; see Figure 15 and no significant interaction between exercise pattern and intensity $F_{(3,66)} = 0.316$, $p = 0.316$; see Figure 16. Findings for CA2/3 and DG were similar to those of CA1, in which there were significant main effects for exercise pattern (see Table 2)

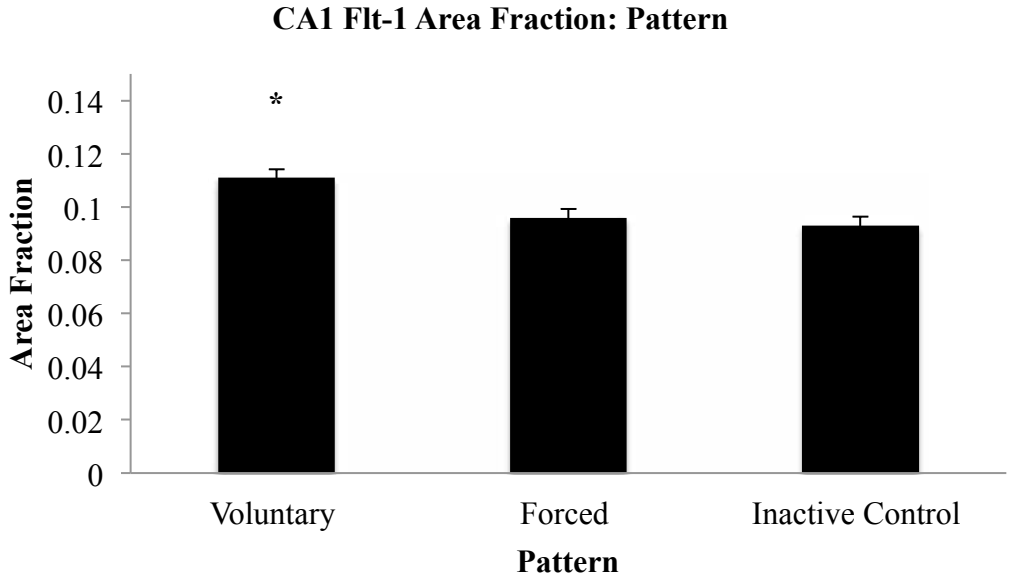

Figure 14: Flt-1 expression based on pattern in CA1. Voluntary exercise* had a significantly greater expression of Flt-1 compared to both forced ($p = 0.003$) and inactive control ($p = 0.0003$).

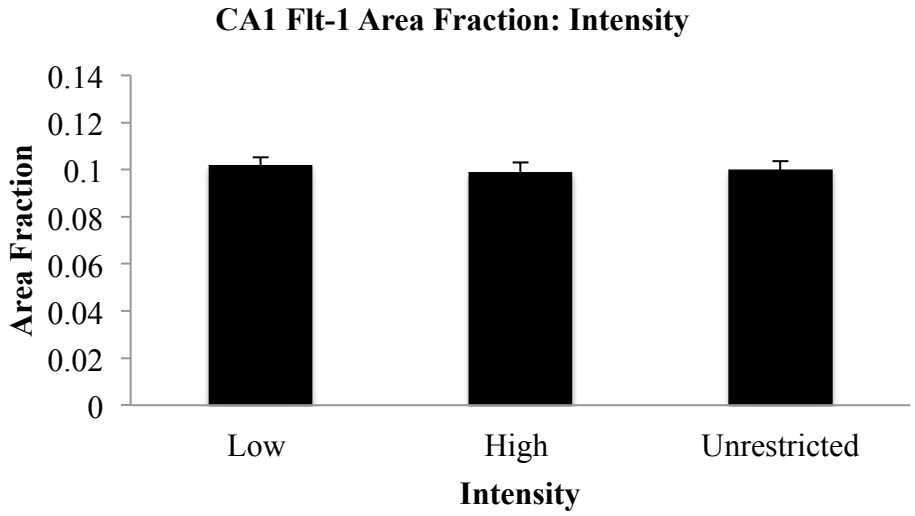

Figure 15: Flt-1 expression based on intensity in CA1. There were no significant differences between intensities ($p = 0.688$).

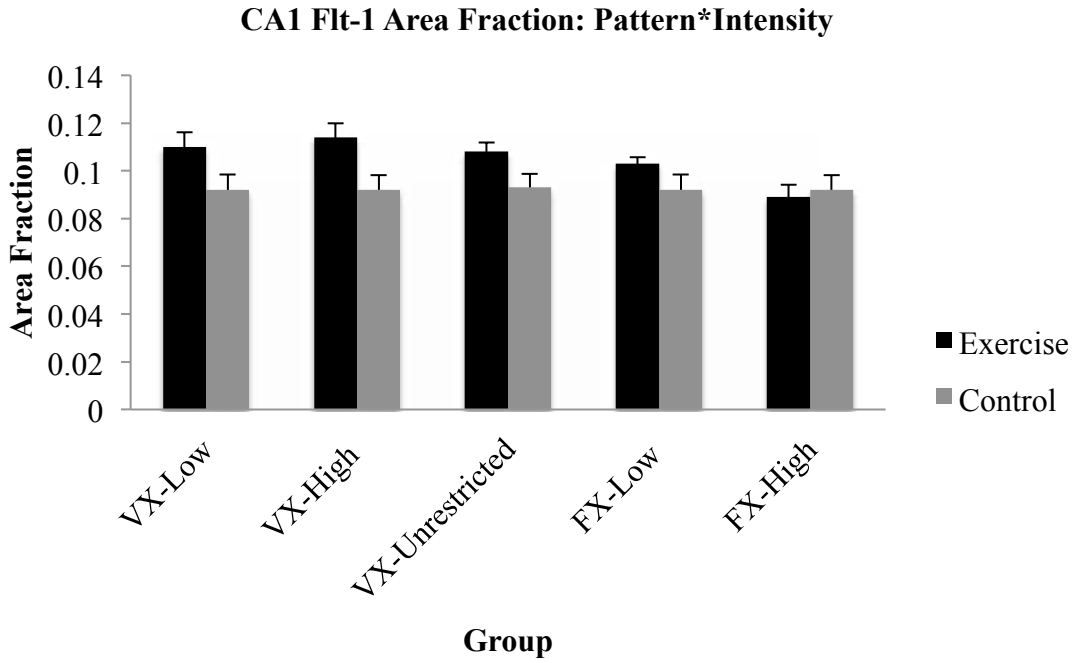

Figure 16: Flt-1 expression based on pattern and intensity in CA1. There was no significant interaction between exercise pattern and intensity ($p = 0.316$).

Table 2

Mean (SEM) and Statistical Significance for Flt-1 Area Fractions: Pattern

	Hippocampal Region		
Pattern	CA1	CA2/3	DG
Voluntary	0.111 (0.003)*	0.105 (0.003)*	0.114 (0.003)*
Forced	0.096 (0.003)	0.096 (0.004)	0.100 (0.004)
Inactive Control	0.093 (0.003)	0.096 (0.003)	0.101 (0.005)
Sig. *(p)*	0.00009	0.038	0.021

Flk-1 and Flt-1 Expression: Cerebellar Paramedian Lobule

Tissue from 72 of the 74 animals was used in cerebellar analyses. Tissue sections from two inactive control animals were damaged during immunohistochemistry, preventing imaging and quantification. For these two animals, group means were substituted for statistical analyses. For both Flk-1 and Flt-1, analyses were completed for the whole paramedian lobule, and for two separate regions of interest within the paramedian lobule: the molecular layer and the granular/Purkinje layers. Each analysis involved running a two-way ANOVA with pattern (forced, voluntary, or inactive control) and intensity (high, low, or unrestricted) as independent variables and either Flk-1 and Flt-1 area fractions as dependent variables.

Flk-1. For the whole paramedian lobule, a two-way ANOVA indicated a significant main effect for intensity $F_{(2,66)} = 4.54$, $p = 0.014$; see Figure 17. Post hoc tests (LSD) showed significantly higher expressions of Flk-1 in low intensity groups ($M = 0.197$, $SEM = 0.008$) compared to both high ($M = 0.173$, $SEM = 0.007$; $p = 0.008$) and unrestricted groups ($M = 0.172$, $SEM = 0.007$; $p = 0.013$). There was no significant difference between high ($M = 0.173$, $SEM =$

0.007) and unrestricted groups ($M = 0.172$, $SEM = 0.007$; $p = 0.897$). There was also a significant interaction between exercise pattern and intensity $F_{(3,66)} = 6.132$, $p = 0.001$; see Figure 18. Pairwise comparisons showed a significant difference between FX-L ($M = 0.217$, $SEM = 0.010$) and FX-H ($M = 0.141$, $SEM = 0.005$; $p = 0.00006$). Pairwise comparisons indicated no significant differences between other groups. See Table 3 for means and standard error of the means for all other groups. There was no main effect for exercise pattern $F_{(2,66)} = 0.489$, $p = 0.615$; see Figure 19.

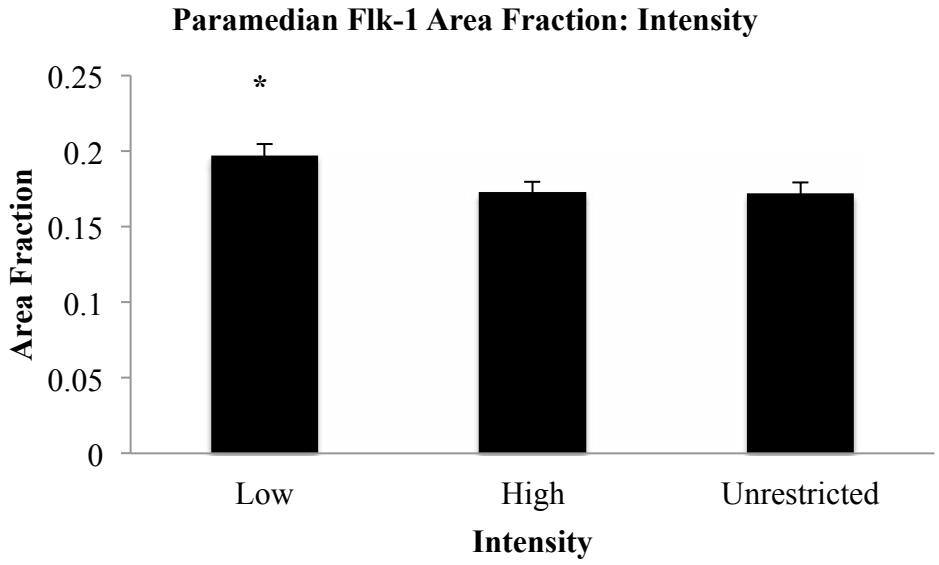

Figure 17: Paramedian lobule Flk-1 expression based on intensity. Low intensity* had a significantly higher expression of Flt-1 compared to both high ($p = 0.008$) and unrestricted ($p = 0.013$).

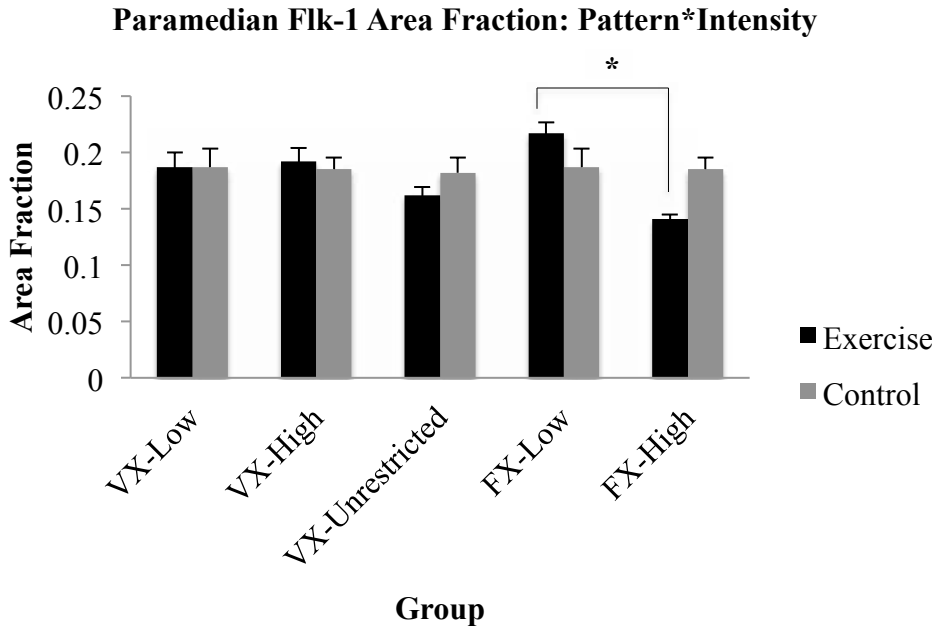

Figure 18: Paramedian lobule Flk-1 expression for pattern and intensity. There was a significant interaction ($p = 0.001$); FX-H* had significantly lower Flk-1 expression compared to FX-L ($p = 0.00006$).

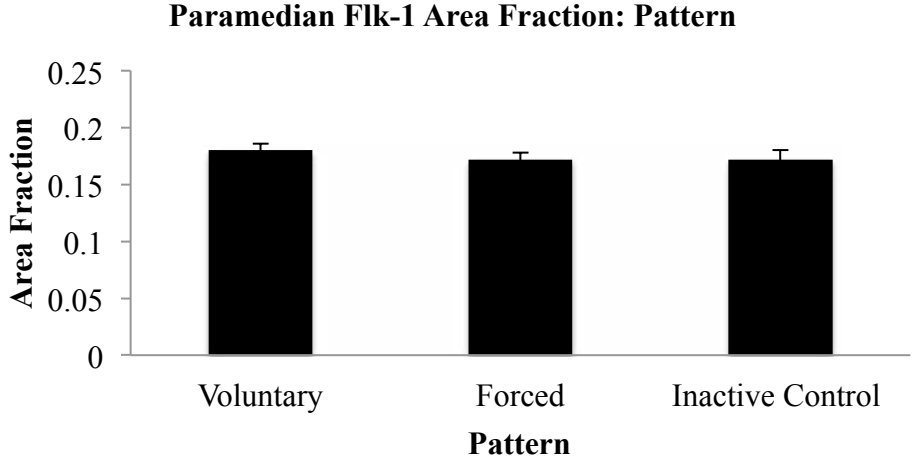

Figure 19: Paramedian lobule Flk-1 expression based on pattern. There were no significant differences between patterns ($p = 0.615$).

Table 3

*Mean and SEM for Paramedian Flk-1 Area Fractions: Pattern * Intensity*

Group	*Mean*	*SEM*
VX-L	0.187	0.013
VX-H	0.192	0.012
VX-U	0.162	0.007
FX-L	0.217	0.010
FX-H	0.182	0.004
IC-L	0.187	0.016
IC-H	0.185	0.010
IC-U	0.182	0.012

For Flk-1, findings for both the molecular and granular/Purkinje cell layers were similar to those of the whole paramedian lobule. For the molecular layer, there was a significant main effect for intensity $F_{(2,66)} = 3.158$, $p = 0.049$. There was also a significant main effect for intensity in the granular/Purkinje cell layers $F_{(2,66)} = 3.514$, $p = 0.035$. For the molecular layer, post hoc tests (LSD) indicated the low intensity groups had a significantly greater expression of Flk-1, compared to the high intensity groups, $p = 0.010$. In the granular/Purkinje layers, post hoc tests (LSD) showed that low intensity was significantly different from both high, $p = 0.015$ and unrestricted, $p = 0.018$ intensities (see Table 4 for means and standard errors). The interaction between pattern and intensity was also significant for the molecular layer $F_{(3,66)} = 4.337$, $p = 0.008$, and granular/Purkinje layers $F_{(3,66)} = 5.045$, $p = 0.003$. Like in the whole paramedian lobule, pairwise comparisons indicated only significant differences between FX-L and FX-H for

both the molecular layer, $p = 0.00005$; see Figure 20 and granular/Purkinje layers, $p = 0.00004$; see Figure 21. There was no significant difference in exercise pattern for the molecular layer $F_{(2,66)} = 0.860$, $p = 0.428$ or the granular/Purkinje layers $F_{(2,66)} = 0.138$, $p = 0.871$.

Table 4

Mean (SEM) and Statistical Significance for Flk-1 Area Fractions: Intensity

	Paramedian Lobule Layer	
Intensity	Molecular	Granular/Purkinje
Low	0.197 (0.008)	0.199 (0.008)
High	0.172 (0.008)	0.175 (0.009)
Unrestricted	0.189 (0.010)	0.173 (0.008)
Sig. *(p)*	0.049	0.035

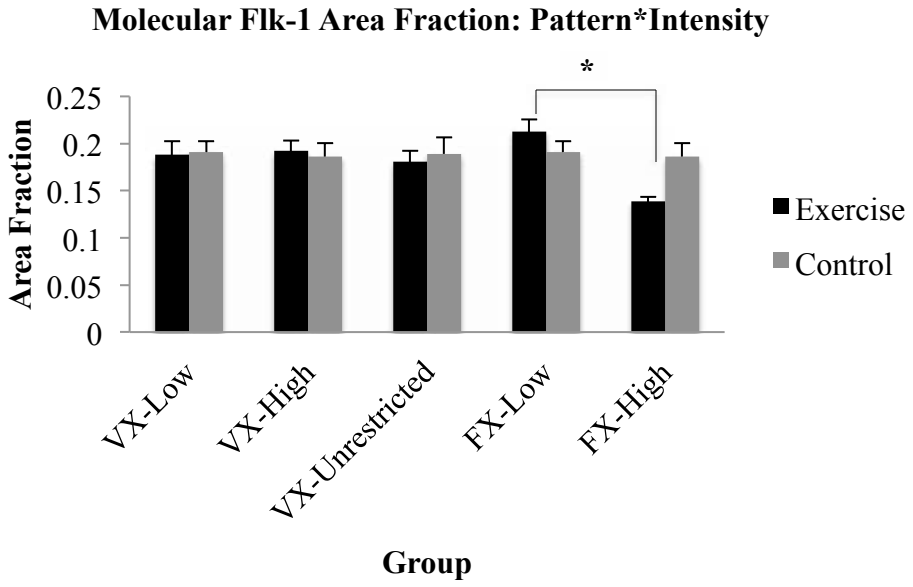

Figure 20: Molecular layer Flk-1 expression for pattern and intensity. There was a significant interaction ($p = 0.008$); FX-H* had significantly lower Flk-1 expression compared to FX-L ($p = 0.00005$).

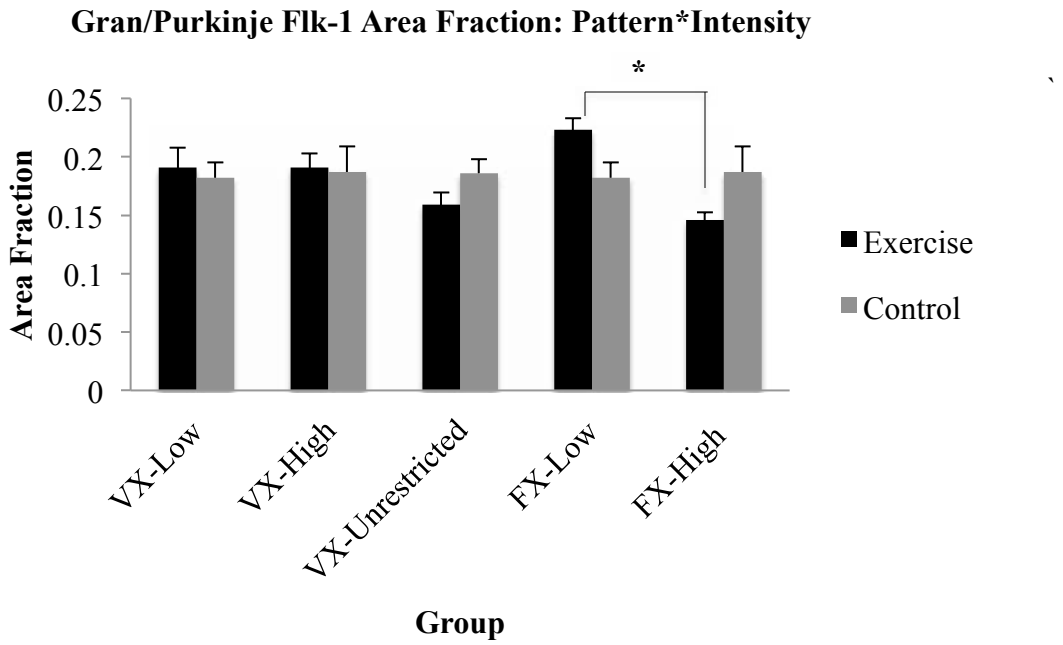

Figure 21: Granular/Purkinje layers Flk-1 expression for pattern and intensity (p = 0.003). There was a significant interaction (p = 0.003); FX-L* had significantly greater Flk-1 expression compared to FX-H (p = 0.00004).

Flt-1. The pattern of expression for Flt-1 in the paramedian lobule differed from that of Flk-1. For the whole paramedian lobule, there was a significant main effect for pattern $F_{(2,66)}$ = 9.848, p = 0.0002; see Figure 21. Post hoc tests (LSD) indicated Flt-1 expression was significantly higher in voluntary exercise groups (M = 0.131, SEM = 0.005) compared to forced exercise groups (M = 0.109, SEM = 0.005; p = 0.003). Flt-1 was significantly lower in voluntary exercise groups (M = 0.131, SEM = 0.005) compared to inactive control groups (M= 0.147, SEM = 0.005; p = 0.025). Furthermore, Flt-1 expression was significantly lower in forced exercise groups (M = 0.109, SEM = 0.005) compared to inactive control groups (M= 0.147, SEM= 0.005; p= 0.00004). Unlike for Flk-1, there was no significant main effect for intensity F (2,66) =

1.945, $p = 0.151$; see Figure 22, and no significant interaction between pattern and intensity $F(3,66) = 0.757$, $p = 0.522$; see Figure 23.

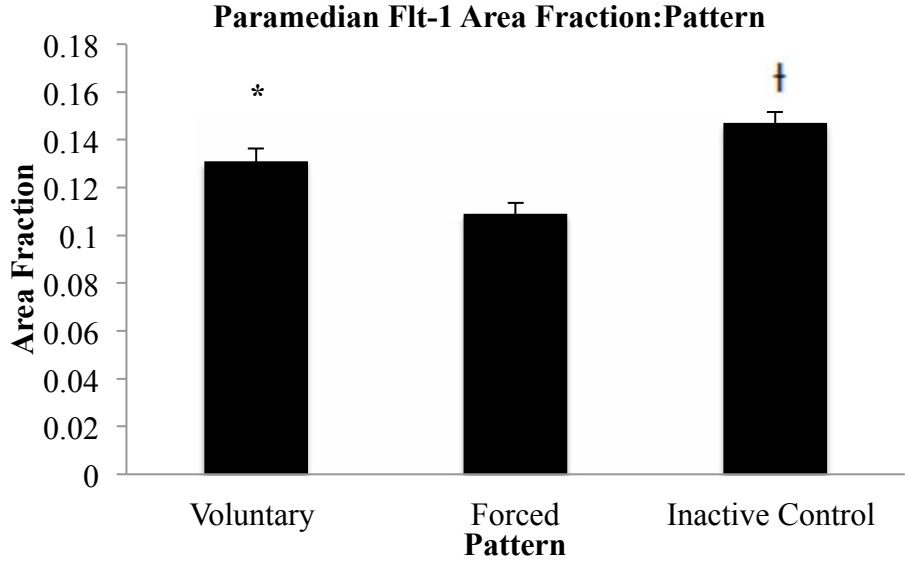

Figure 22: Paramedian lobule Flt-1 expression based on pattern. Inactive control† was significantly higher than both forced ($p = 0.00004$) and voluntary ($p = 0.025$), and voluntary* exercise was significantly higher than forced exercise ($p = 0.003$).

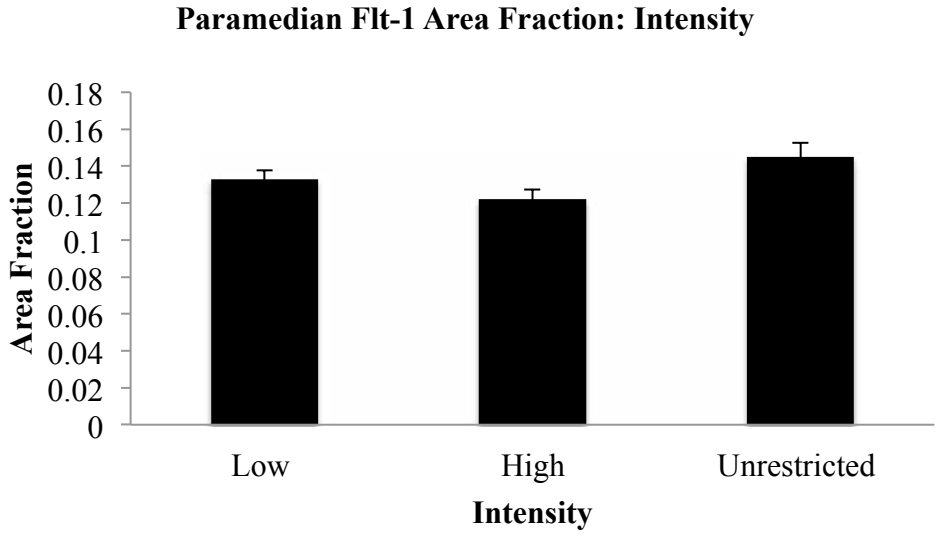

Figure 23: Paramedian lobule Flt-1 expression based on intensity. There were no significant differences between intensities ($p = 0.151$).

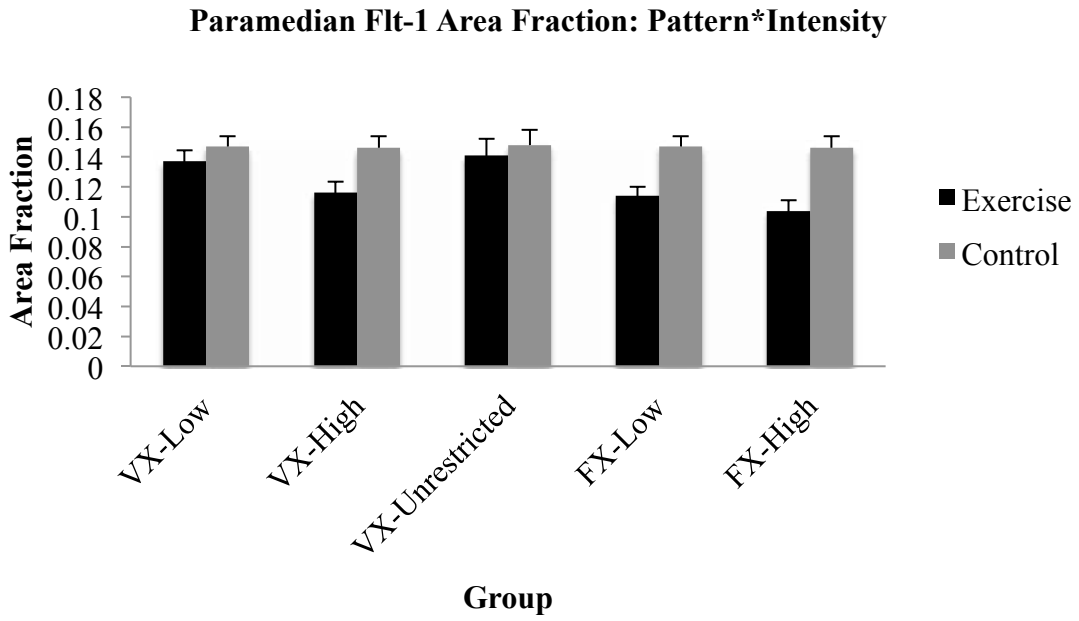

Figure 24: Paramedian lobule Flt-1 expression based on both pattern and intensity. There was no significant interaction between pattern and intensity ($p = 0.522$).

Like in the whole paramedian lobule, there was a significant main effect for pattern in both the molecular layer $F_{(2,66)} = 8.838$, $p = 0.00004$; see Figure 24, and the granular/Purkinje layers $F_{(2,66)} = 7.034$, $p = 0.001$; see Figure 25. For the molecular layer, post hoc tests (LSD) showed Flt-1 expression in the forced exercise groups ($M = 0.105$, $SEM = 0.004$) were significantly lower than the inactive control groups ($M = 0.146$, $SEM = 0.005$; $p = 0.0003$). Forced exercise groups ($M = 0.105$, $SEM = 0.004$) were also significantly lower than voluntary exercise groups ($M = 0.144$, $SEM = 0.007$; $p = 0.0003$). However, unlike the paramedian lobule as a whole, voluntary exercise groups ($M = 0.144$, $SEM = 0.007$) were not significantly different from inactive control groups ($M = 0.146$, $SEM = 0.005$; $p = 0.779$).

In the granular/Purkinje layers, follow-up post hoc tests (LSD) for the significant main effect of pattern yielded slightly different findings in comparison to the molecular layer. Flt-1

expression was significantly higher in inactive control groups (M= 0.151, SEM= 0.004) compared to *both* voluntary exercise groups (M= 0.126, SEM= 0.005; p= 0.003) and forced exercise groups (M= 0.116, SEM= 0.005; p = 0.0002). Unlike for the molecular layer and the whole paramedian lobule, in the granular/Purkinje layers, there was no significant difference between forced exercise groups (M = 0.116, SEM = 0.005) and voluntary exercise groups (M = 0.126, SEM = 0.005; p = 0.219).

Similar to the whole paramedian lobule, there were no significant main effects for intensity in the molecular layer $F_{(2,66)} = 0.700$, $p = 0.50$ or the granular/Purkinje layers $F_{(2,66)} = 0.942$, $p = 0.395$. There was also no significant interaction between pattern and intensity in either the molecular layer $F_{(3,66)} = 0.534$, $p = 0.661$ or the granular/Purkinje layers $F_{(3,66)} = 0.623$, $p = 0.603$.

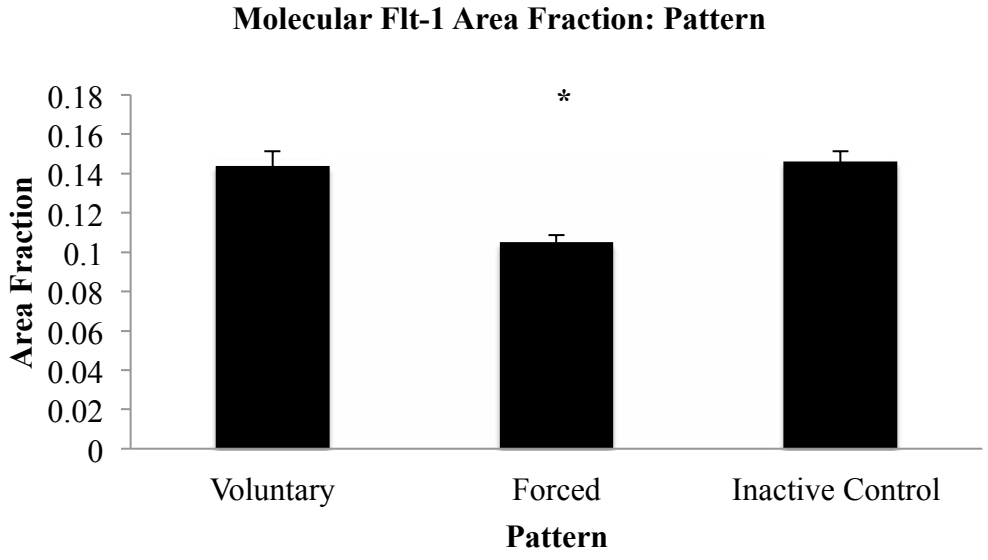

Figure 25: Molecular layer Flt-1 expression based on pattern. Forced exercise* was significantly lower than both voluntary (p = 0.007) and inactive control (p = 0.0002).

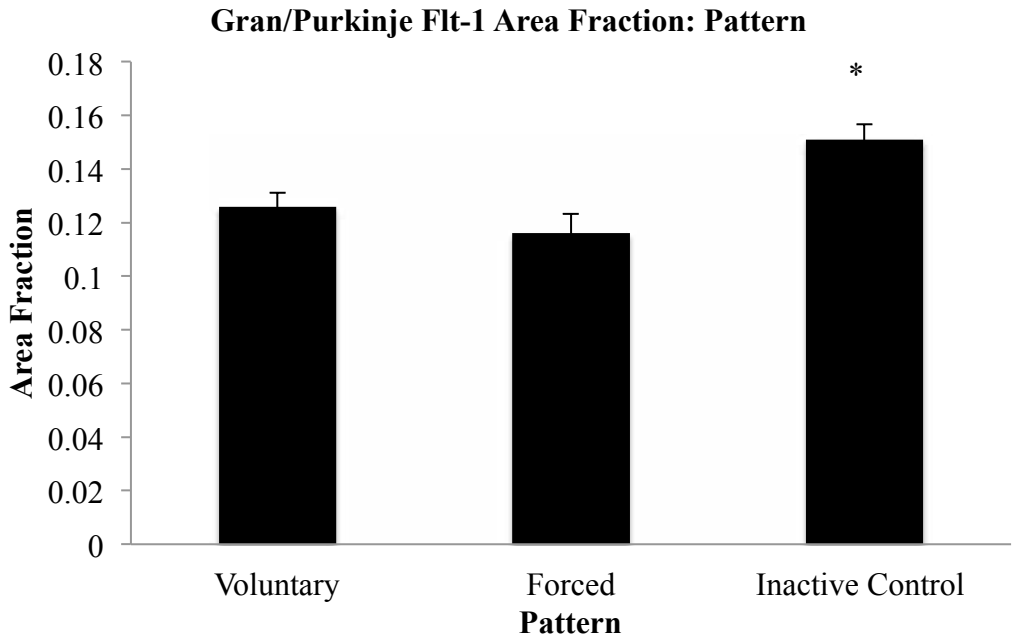

Figure 26: Granular/Purkinje layers Flt-1 expression based on pattern. Inactive control* was significantly higher than both voluntary ($p = 0.003$) and forced exercise ($p = 0.0002$).

Discussion

The goal of this experiment was to investigate the effects of exercise pattern and intensity on the expression of Flk-1 and Flt-1 in the hippocampus and cerebellum. The majority of previous studies that have examined differences in pattern or intensity have focused on neuronal, rather than vascular changes. This study addressed this deficit by measuring the expression of Flk-1 and Flt-1, two receptors that are critical in the regulation of angiogenesis. Furthermore, this experiment aimed to clarify the effects of exercise pattern and intensity on angiogenesis after an acute bout of exercise. Past experiments have quantified neurovascular changes after several days to weeks.

It was hypothesized that VX-U would display the highest expression of Flk-1 and Flt-1 receptors, followed by VX-H, VX-L, and FX-L. Animals in the FX-H group were expected to have the lowest expression of Flk-1 and Flt-1 receptors. Both the hippocampus and cerebellum

were expected to display this same pattern of expression. These hypotheses were, in part, supported. In the hippocampus, although there were no significant differences induced in labeling by the intensity of exercise, animals that engaged in voluntary exercise had significantly higher expressions of Flk-1 and Flt-1 compared to both forced exercise and inactive control animals. These results did not hold true in the cerebellum. Findings in the cerebellum, although interesting, did not support the original hypotheses. For Flk-1, there was no significant effect for exercise pattern. However, there was a significant difference in the labeling of these receptors based on the intensity of exercise. Animals that engaged in low intensity exercise displayed a significantly higher expression of Flk-1 compared to both high and unrestricted groups. Pairwise comparisons indicated a significant difference in Flk-1 expression between FX-L and FX-H. For Flt-1 there was a suppression in exercising animals. Both forced and voluntary exercise produced significantly lower Flt-1 expression compared to inactive controls. These findings did not support the hypothesis, which had originally expected elevations in Flt-1.

Hippocampus

In the hippocampus, both Flk-1 and Flt-1 were elevated in voluntary exercise, compared to forced exercise and inactive control. Our results correspond with those that have found that voluntary exercise produces a greater neurogenic response, compared to forced exercise. For example, following cerebral ischemia, Ke et al. (2011) found that seven days of voluntary exercise increased BDNF expression in the hippocampus significantly more than forced exercise and inactive control groups. Similarly, following cerebral ischemia, voluntary exercise for five days increased the survival of newly birthed neurons in the dentate gyrus; forced swim exercise did not (Luo et al., 2007). Our work adds to this literature, suggesting that not only does

voluntary exercise enhance neurovascular changes after a few days of exercise, but also this difference between forced and voluntary exercise is apparent after only one day of exercise.

Our findings, at first glance, seem to differ from those of Hayes et al. (2008), Kinni et al. (2011), and Leasure and Jones (2008). All of these studies found forced exercise produced greater neurovascular effects than voluntary exercise. Hayes et al. (2008) showed that three weeks of forced treadmill exercise, prior to exposure to cerebral ischemia, was more neuroprotective than voluntary exercise. Kinni and colleagues (2011) investigated the effects of exercise pattern on cerebral metabolism, finding that after three weeks of exercise HIF-1α mRNA and protein were significantly elevated in the forced exercise group, compared to both voluntary exercise and inactive control groups. Leasure and Jones (2008) used a forced exercise paradigm (motorized wheel) most similar to the one used in our study. They found that after eight weeks of exercise, in which forced and voluntary exercise animals ran the same distances, both forced and voluntary exercise groups had an increased percentage of neuronal progenitor cells, but this elevation was significantly greater in the forced exercise group.

Explanations. There are several possible explanations as to why our study complements the findings of some, but differs from the conclusions of others. This includes differences in speed of the exercise, strategies used to quantify neurovascular changes, how strenuous the forced exercise paradigm was, the duration of the exercise paradigm, and the levels of stress associated with the exercise.

First, one possible explanation for differences between our findings and those of Hayes et al. (2008) and Kinni et al. (2011) is the speed used in the forced exercise paradigm. Hayes et al. (2008) and Kinni et al. (2011) used the forced treadmill exercise in which animals ran at speeds of 30 m/min. In our study, animals ran at a slower speed of ~9 m/min on a motorized wheel. It

may be that for forced exercise to produce a high degree of neurovascular change it must be done at a rapid speed. However, this explanation does not seem highly plausible. According to the results from Lou et al. (2008), low intensity forced exercise (completed at slower speeds) was more neuroprotective than high intensity forced exercise (completed at rapid speeds).

Another possible explanation for the differences between voluntary and forced exercise relates to whether the measurement strategy used to capture changes in angiogenesis or neurogenesis quantifies actual changes in neuron and vessel, or if the measurement strategy quantifies more transient markers, such as growth factors. For example, Kinni et al. (2011) found that HIF-1α was higher in forced rather than voluntary exercise after eight weeks and thus had a greater angiogenic effect. Alternatively, these findings may suggest changes in forced exercise happen more gradually. It may be that the vascular system has already adapted in the voluntary exercise groups and HIF-1α is returning to baseline levels. HIF-1α may only be elevated in forced exercise groups because the response is lagging behind. Thus, if actual vessels were quantified, there could be more in voluntary exercise groups compared to forced exercise groups. Although this may explain differences in some studies, this proposition does not always hold true. Hayes et al. (2008) measured brain infarct volume, rather than the expression of transient markers, and found forced exercise to significantly reduce this volume in comparison to voluntary exercise.

A third possible explanation for differences between forced and voluntary exercise is that forced exercise is more strenuous than voluntary exercise. During forced exercise, animals are required to run for up to one hour with no breaks, and this may cause greater metabolic deficits compared to animals that engage in voluntary exercise on and off throughout the day (Kinni et al., 2011). Based on our work, and those of Lou et al. (2008), this suggestion also does not seem

highly plausible. In our study, animals engaged in forced exercise at either a low (500 meter) or high (1,000 meter) intensity. In both cases, neither the low nor the high intensity forced exercise groups had elevations of Flk-1 or Flt-1, and, even more importantly, there were no significant differences between the FX-L and FX-H groups. The averages between these two groups were similar for both Flk-1 and Flt-1 expression, regardless of intensity. If greater metabolic demands increase the expression of angiogenic factors, the FX-H group, which ran twice as far, should have displayed a differential elevation in Flk-1 or Flt-1. This was not apparent. Additionally, if this were accurate, Lou et al. (2008), the study previously mentioned, which investigated intensity by manipulating speed, would have found that the high intensity group exhibited signs of greater neuroprotection than the low intensity group. They found the opposite, suggesting that greater metabolic demands are not advantageous.

Another, more probable explanation relates to the duration of the exercise paradigm. Hayes et al. (2008) and Kinni et al., (2011) had animals run for three weeks and Leasure & Jones (2008) had animals run for eight weeks. Our study had animals run for 24 hours, which is considerably shorter. Interestingly, other studies that also found more beneficial effects in voluntary compared to forced exercise also had animals run for shorter durations including five (Luo et al., 2007) and seven (Ke et al., 2011) days. Therefore, it is possible that voluntary exercise produces more rapid neurovascular changes compared to forced exercise, which are apparent during the first week of exercise. However, after nearly a month or more of forced exercise, it may be that *over time*, forced exercise produces greater neurovascular changes than voluntary.

A further explanation important to address are the effects of stress, associated particularly with forced exercise. Although forced exercise is not used to induce stress, whether or not stress

is an artifact of this paradigm is debatable. This paradigm has undertones of traditional stress paradigms, including repeated exposure, electric shock, and lack of control. Also, it is clear that hippocampal angiogenesis and neurogenesis are vulnerable to the effects of stress (Czeh Aburmaria, Rygula & Fuchs, 2010; Heine et al., 2005; Elkstrand, Hellsten, & Tingstrom, 2008). Repeated stress exposure decreases microvessel density throughout the hippocampus by 30% (Czeh et al., 2010). Heine and colleagues (2005) found the effects of stress on angiogenesis mediate the effects of stress on neurogenesis. Chronic stress significantly decreased the number of proliferating neuronal cells in the dentate gyrus, and the portion of newborn cells most anatomically intermingled with the microvasculature was affected most. Increases in VEGF and Flk-1 protein were associated with this effect. Elkstrand et al. (2008) found that treatment with corticosterone directly reduced the number of BrdU/RECA-1 (an endothelial cell marker) positive cells in the hippocampus.

In sum, the literature suggests that the effects of stress can retard and even suppress angiogenesis and neurogenesis in the hippocampus. However, how stress acts a factor in forced exercise remains somewhat unclear. Following forced exercise some studies have found elevations in corticosterone (Ke et al., 2011; Hayes et al., 2008) and others have not (Leasure & Jones, 2008). Furthermore, interpretations regarding the effects of elevated corticosterone following forced exercise vary. Some argue that high corticosterone levels are advantageous (Hayes et al., 2008; Kinni et al., 2011) and others suggest the effects are harmful (Ke et al., 2011) to exercise induced neurovascular changes. It is also important to note that forced exercise still benefits animals that experience traditional chronic stress paradigms. When animals engaged in forced exercise concomitantly with chronic stress for two weeks, there was a decrease in depression-like behavior and an increase in both blood vessel density and neuronal survival,

compared to animals experiencing stress alone (Kuichi et al., 2012). Furthermore, when the VEGF/Flk-1 signaling pathway was blocked, exercise no longer diminished the effects of stress (Kuichi et al., 2012).

Habituation of the stress response is a highly plausible explanation as to why the effects of stress may not affect neurovascular changes associated with forced exercise. It has been demonstrated that repeated exposure to the same stressor results in decreases in the stress response at the behavioral (Kearns & Spencer, 2013) and molecular (Grissom, Iyer, Vining, & Bhatnager, 2007) level. Grissom and colleagues (2007) found that when rats were chronically restrained for seven days in the same context, there was a decrease in corticosterone and ACTH in comparison to animals that had been restrained for the first time (acute stressor). Similarly, Kearns and Spencer (2013) found that after rats experienced restraint stress, daily for the same duration, there was a significant decrease in corticosterone after four days. This was accompanied by a decrease in struggling behavior typically associated with restraint stress (Kearns & Spencer, 2013).

This habituation of the stress response may underlie the explanation previously discussed: duration of the exercise paradigm. As previously mentioned, forced exercise, after weeks to months, seems to produce more robust neurovascular changes compared to voluntary exercise. However, voluntary exercise is more advantageous in the short-term. It may be that after animals experience the same forced exercise paradigm day after day, it no longer elicits a large stress response. Furthermore, this 4-7 day habituation time point, in which the stress response decreases, coincides with studies that have seen greater benefits of forced exercise. Forced exercise is more advantageous than voluntary in regards to the neurovascular system when changes are quantified after two or more weeks of exercise (Kuichi et al., 2012; Leasure &

Jones, 2008, Hayes et al., 2008). This is likely enough time for animals to habituate to the effects of stress associated with forced exercise. Intriguingly, studies that find the most benefit associated with voluntary exercise compared to forced exercise have measured these changes during the first week of exercise (Ke et al., 2011; Luo et al., 2007). This includes our study, which measured Flk-1 and Flt-1 expression after a single bout of exercise. At this time, corticosterone may be exerting its greatest effects, as the animal has not habituated to the effects of stress associated with forced exercise. It is likely that stress could be delaying the upregulation of Flk-1 and Flt-1 in the forced exercise group. However, had we measured after several weeks of exercise, we may have seen a greater upregulation of Flk-1 and Flt-1 in forced, not voluntary exercise.

Cerebellum

Unlike the hippocampus, the cerebellar paramedian lobule has been primarily implicated in motor movement and motor learning (Black et al., 1990). The functional difference between these two structures likely explains the differences in our results. The hippocampus is involved in numerous cognitive functions, including the regulation of emotion and memory (Czeh et al., 2010; van Praag et al., 2007). Angiogenesis that occurs in the hippocampus following exercise primarily provides metabolic support for increased neurogenesis, a process also enhanced with exercise. The cerebellum, particularly the paramedian lobule, has been primarily implicated as a motor region. In the cerebellum, Issacs et al. (1992) suggest that angiogenesis is a direct response to the metabolic demands associated with functional activity. Angiogenesis functions as a way to decrease diffusional distance when metabolic demands increase.

In our study, findings in the cerebellum indicated that forced exercise, at a low intensity produces the greatest amount of Flk-1 labeling. Interestingly, for Flt-1, there was a suppression

in both exercising groups, with forced exercise being significantly lower than voluntary. To our knowledge, this is the first study to investigate the effects of exercise pattern and intensity on the expression of Flk-1 and Flt-1 in the cerebellum. It is clear that both forced and voluntary exercise produce angiogenesis in the cerebellum (Black et al., 1990; Isaacs et al., 1992), but whether forced and voluntary exercise has differential effects has not been investigated. Furthermore, no studies have investigated the effects of intensity on the cerebellum. After one month of exercise, Black et al. (1990) found increased capillary density in both voluntary and forced exercise animals. They attributed this to the increased metabolic activity associated with exercise. Animals that engaged in an acrobatic learning task had increased synaptogenesis, rather than capillary density. This effect was due to motor learning, rather than motor activity, which is associated with exercise (Black et al., 1990). Increased motor activity is the primary factor that drives angiogenesis in the cerebellum (Black et al., 1990; Issacs et al., 1992).

The type of motor activity that causes the greatest increase in angiogenesis in the cerebellum has not been investigated. In our study, the voluntary exercise animals, at all intensities, did not greatly differ from inactive controls in regards to Flk-1 labeling. It was only the FX-L group that showed elevated Flk-1 area fractions. It may be that the strenuous, repeated motor activity associated with forced exercise contributed to this difference (Kinni et al., 2011). Voluntary exercise animals, on average, took much longer to complete the required exercise regimens as they engaged in exercise intermittently for several hours. It may be that forced exercise, which involved repeated motor activity for one hour, creates a greater metabolic deficit and thus triggered a greater angiogenic response in the FX-L group.

Our findings also showed a significant difference between the FX-L and FX-H groups in regards to Flk-1 expression. Only the FX-L group displayed elevations in Flk-1; FX-H displayed

very low Flk-1 expression. Outside of the cerebellum, some findings indicate that low intensity forced exercise produces greater effects than high intensity forced exercise in the hippocampus. As previously discussed, Lou and colleagues (2008) found that their low intensity group had higher expression of BDNF, VEGF, and Flk-1 mRNA, compared to the high intensity group. Lou et al., (2008), assessed the effects of intensity by varying the speed of the exercise; all animals ran for the same duration. Animals in the low intensity group ran at speeds between 5-11m/min. This is similar to the speed of ~9m/min used in our study. This study defined intensity in terms of speed. Our study defined intensity in terms of distance. However, although intensity was defined somewhat differently, it still had an impact on Flk-1 expression. Animals that engaged in high intensity forced exercise, but not low intensity forced exercise, had decreased Flk-1 expression. Lou et al. (2008) suggest that high intensity exercise may alter energy metabolism the brain, in a way that reduces the expression of growth factors. Furthermore, human studies suggest that exercise done at a high intensity decreases brain glucose uptake. Kemppainen et al. (2005) found that brain glucose uptake was reduced by 27% in people that engaged in the highest intensity forced exercise condition. Interestingly, in the low intensity group, glucose uptake was 22% higher in the cerebellum. It is possible that similar effects, in regards to energy metabolism, occurred in the cerebellum in our forced exercise groups.

Another interesting finding in the cerebellar paramedian lobule was the suppression of Flt-1 in both forced and voluntary exercise groups. Furthermore, levels were significantly lower in forced compared to voluntary exercise. This suppression may be related to the function of the Flt-1 receptor. Flt-1 is important for the organization of the vascular system (Shibuya et al., 1990; Fong et al., 1995). However, it also has a critical role in preventing vascular overgrowth. For example, Flt-1 has been found to negatively regulate blood vessel formation during

development (Roberts et al., 2004). It does this, in part, by preventing endothelial cell division. During embryonic development, Flt-1 has been shown to interrupt Flk-1 signaling by affecting the degree of tyrosine phosphorylation of the Flk-1 receptor (Roberts et al., 2004). Furthermore, Flt-1 has been suggested to act as a "sink," sequestering the VEGF that is available to bind to Flk-1 (Robert et al., 2004; Kappas et al., 2008). This action allows Flt-1 to regulate signaling through Flk-1 (Kappas et al., 2008). Therefore, when Flt-1 is absent, or downregulated, there would be more VEGF available to bind to Flk-1. Signaling through Flk-1 is the first step in angiogenesis, as it leads to the proliferation of endothelial cells. Thus, it may be that these two receptors work somewhat in opposition.

In a state of high metabolic demand, the organism will want to mount an angiogenic response as rapidly as possible in effort to adapt. Thus, it may be advantageous to suppress expression of Flt-1 because binding of VEGF to this receptor may negatively modulate proliferation and, in addition, Flt-1 may directly sequester VEGF, decreasing its availability to Flk-1. Our results suggest this effect may have been occurring the in the cerebellum. Although we only saw elevations of Flk-1 in the FX-L group, it is likely that there will soon be an increase in Flk-1 expression in all exercising groups. Therefore, it may be advantageous to downregulate Flt-1 during a time where Flk-1 would be expected to be rising. This would allow for the most robust proliferative effect.

Future Directions

It is important to further explore the temporal differences in angiogenesis, in regards to forced vs. voluntary exercise in the hippocampus and cerebellum. A limitation of this study is that all animals were sacrificed 24 hours after the *onset* of exercise. This meant that some animals had been done exercising several hours prior to sacrifice, and other animals engaged in

exercise up until the time of sacrifice. The issue is, that in forced exercise groups, Flk-1 and Flt-1 may have been upregulated immediately following exercise but not hours later. Some evidence exists for this proposition. Ploughman et al. (2007) found that motorized wheel running produces a more rapid increase in BDNF, which was only detected 20 minutes post exercise. Voluntary exercise produced a more lasting upregulation of BDNF still detectable at their final time of measurement, which was two hours post exercise. Therefore, it is possible that forced exercise caused rapid bursts in Flk-1 and Flt-1 expression, that would have only been detectable immediately post exercise. Although it seems that a more lasting increase in the expression of neurovascular factors would be more advantageous, it is still important to clarify whether or not these factors are expressed, to any degree, following an acute bout of forced exercise. Future studies should address this by sacrificing animals immediately post exercise, or consistently sacrificing animals a certain number of hours post exercise.

Again, as mentioned previously, it will also be important to understand this temporal difference between voluntary and forced exercise in relation to the effects of stress and the habituation of the stress response. Future studies should address this issue by having animals engage in forced and voluntary exercise not only for an acute duration such as 24 hours, but also for a longer duration such as one month. In parallel, corticosterone levels should be measured.

A final issue future studies should address is whether or not Flk-1 and Flt-1 is specifically labeling endothelial cells. In our study, we looked only for Flk-1 and Flt-1 labeled receptors regardless of their source of origin. In theory, these receptors could have been expressed on endothelial, glial, or neuronal cell types. Although Flk-1 and Flt-1 are primarily implicated in angiogenesis, some studies have suggested VEGF has direct roles in neurogenesis (Cao et al., 2004). To be certain expression of Flk-1 and Flt-1 is predominately being increased on

endothelial cells, and thus regulating angiogenesis, a double-labeling experiment is an important future step.

Conclusions and Implications

Our study has shown that particular exercise patterns and intensities do not affect the brain in a universal manner. We have shown a differential effect between the hippocampus and cerebellum. In the hippocampus, forced exercise, regardless of intensity, produces no increase in Flk-1 and Flt-1. In contrast, voluntary exercise, at low, high, or unrestricted intensities produce significant increases in both of these receptors. In the cerebellum, we have demonstrated that only low intensity exercise produces significantly greater expressions of Flk-1. This was especially apparent in the forced exercise groups, with FX-H displaying significantly less Flk-1 labeling than FX-L. We also showed a suppression of Flt-1 in all exercise groups, compared to inactive controls. Furthermore, we have shown that changes in Flk-1 and Flt-1 expression, two high affinity receptors for VEGF, can be detected after an acute bout of exercise.

These findings have important implications, particularly in the study of brain injury and recovery. Following brain damage, such as an ischemic insult, it is important that angiogenesis is upregulated rapidly, as its increase is strongly correlated with positive functional outcomes (Austin et al., 2004). Angiogenic changes can restore the delivery of oxygen and nutrients to the damaged brain region (Beck & Plate, 2009). Greater microvessel densities surrounding the ischemic border correlates with increased survival in human patients (Krupinski et al., 1994). Our findings add suggest that, depending on the brain region affected, different exercise patterns and intensities may be more advantageous than others in inducing the most robust angiogenic response. Furthermore, these studies suggest that, following an ischemic insult, it may be

important for patients to begin rehabilitation using a more flexible voluntary regimen, and, after a several days, switch to a more prescribed, forced regimen.

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