August 2013

Exercise-induced Hypoxia, Angiogenesis, and Behavioral Flexibility in the Adult Rat

Kiersten Lee Berggren
University of Wisconsin-Milwaukee

EXERCISE-INDUCED HYPOXIA, ANGIOGENESIS, AND BEHAVIORAL FLEXIBILITY IN THE ADULT RAT

by

Kiersten Lee Berggren

A Dissertation Submitted in

Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy

in Psychology

at

The University of Wisconsin-Milwaukee

August 2013

ABSTRACT
EXERCISE-INDUCED HYPOXIA, ANGIOGENESIS, AND BEHAVIORAL FLEXIBILITY IN THE ADULT RAT

by

Kiersten Lee Berggren

The University of Wisconsin – Milwaukee, 2013
Under the Supervision of Professor Rodney A. Swain

Exercise induces a myriad of effects on the brain from the growth of new capillaries and neurons, to improvements in cognitive performance. Additionally, recent research has shown that commencement of an exercise regimen also causes apoptosis. Therefore, it is possible that exercise-induced increases in oxygen demand cause the brain to transiently experience a state of hypoxia. To investigate this hypothesis, we measured protein levels of hypoxia inducible factor 1 alpha (HIF-1α), a transcription factor known to be upregulated in conditions of hypoxia or ischemia, in animals exposed to a single bout of treadmill exercise. After exercise animals were sacrificed at various time points, ranging from immediately post-exercise to six hours after exercise; we also investigated the effects of long-term exercise on HIF-1α expression. We found that animals sacrificed immediately or four hours after exercise had significantly greater HIF-1α expression in area CA1 of the hippocampus. Because the angiogenic factor vascular endothelial growth factor (VEGF) is a target gene for HIF-1α, in another experiment, we investigated whether HIF-1α expression itself and/or downstream angiogenesis mediate improved performance in the Morris Water Maze (MWM). Animals were exposed to one of five different conditions for one week: forced (FX) or voluntary exercise (VX), inactive condition (IC), or treatment with the HIF-1α agonist dimethyloxylylglycine (DMOG) or vehicle injections (VEH). We found significantly increased HIF-1α expression in area CA1 of the hippocampus in VX, FX, and VEH animals, and significantly increased CA1 capillary density in VX, DMOG, and IC animals. Furthermore, VX, DMOG, and IC animals acquired the task the fastest, but VX, FX,

and DMOG-treated animals performed significantly better than VEH and IC animals during the reacquisition and retention phases of training. Overall, these findings indicate that voluntary exercise or manipulation of HIF-1α expression results in increased capillary density and behavioral flexibility. Therefore, it is possible that HIF-1α serves as a mediator of exercise-induced improvements in cognitive performance, a process that appears to operate by way of increases in capillary density. Because exercise has been shown to be neuroprotective, this research is of potential interest in investigations of ischemia, brain injury, and neurodegeneration.

To

My parents:

Mrs. Mary C. and Mr. Renald R. Berggren,

and to my husband:

Dr. Eric J. Hansen

Thank you for your loving support and encouragement.

"There is 'what is' only when there is no comparing, and to live with 'what is', is to be peaceful." - Bruce Lee

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

EXERCISE-INDUCED HYPOXIA, ANGIOGENESIS, AND BEHAVIORAL FLEXIBILITY IN THE ADULT RAT

Behind cardiovascular diseases and cancer, stroke was the third leading cause of death in the United States in 2006, accounting for 5.7% of total deaths (Heron et al., 2009). The majority of stroke occurrences are classified as ischemic, meaning that blood flow in the brain is significantly decreased or blocked (American Heart Association, 2009); this decrease in blood flow ultimately leads to a decrease in levels of oxygen available to the brain, or hypoxia (Chen, Endler, & Shibasaki, 2009; Marti et al., 2000). Because the brain is plastic, or able to alter itself physiologically in response to internal and environmental changes (Fawcett, Rosser, & Dunnett, 2001), several molecular changes occur in the brain in response to hypoxia (Harten, Ashcroft, & Maxwell, 2010). These biochemical alterations can lead to neuron death via several mechanisms, such as excitotoxicity or the excessive formation and activity of free radicals (Fawcett et al., 2001). Alternately, the neurochemical changes that accompany hypoxia can lead to plastic changes aimed at promoting the survival of neurons, therefore having a neuroprotective effect. One such molecular cascade involves the hypoxia-inducible factors (HIFs), in particular hypoxia-inducible factor 1-alpha (HIF-1α), which is a transcription factor that accumulates in conditions of low oxygen and stimulates the expression of several genes that encode important growth factors (Harten et al., 2010; Mu et al., 2003). These growth factors stimulate biological processes, such as the formation of new blood vessels, in an attempt to compensate for the decreased oxygen availability during and following ischemia (Kaelin & Ratcliffe, 2008; Semenza, 2000b). It is

important to explore the compensatory mechanisms by which the brain protects itself from damage during hypoxia or ischemia, and to develop alternative ways to study these adaptive mechanisms.

The adaptive mechanisms utilized by the brain to maintain oxygen homeostasis during exercise and in conditions of hypoxia involve similar pathways. During ischemia or hypoxia and in response to changes in oxygen and energy supply, several cascades involving HIF-1α are initiated, which result in regulation of genes that encode for potentially neuroprotective proteins such as vascular endothelial growth factor (VEGF), erythropoietin (EPO), heme oxygenase 1 (HO-1), and adrenomedullin (AM) (Harten et al., 2010), that may prevent the cell death that often accompanies stroke or prolonged hypoxic exposure. Similarly, upon the commencement of an exercise regimen, the brain's demand for oxygen and glucose is increased, thereby possibly inducing a mild form of hypoxia in the brain. Kerr and Swain (2011) showed that even short bouts of voluntary exercise were enough to cause apoptosis of neurons in the hippocampus of exercising rats, a process that was followed shortly thereafter by angiogenesis and neurogenesis. Therefore, it is possible that just as in cases of stroke or exposure to hypoxic conditions, exercise may also result in increased expression of hypoxia-induced molecules and thereby be a contributing factor to the initial apoptosis observed following exercise (Kerr and Swain, 2011).

Angiogenesis, or the sprouting of new capillaries from preexisting vessels, is controlled in normal physiological and pathological conditions by VEGF (Ferrara, 1999), and is one physiological response to hypoxia (Chen et al., 2009; Ferrara, 1999; Marti et al., 2000). Similar to stroke or exposure to hypoxia, the commencement of an exercise

regimen has also been shown to induce molecular cascades that result in angiogenesis and the growth of new neurons in the brain. Specifically, exercise has been shown to cause angiogenesis in the cerebellum (Black, Isaacs, Anderson, Alcantara, & Greenough, 1990; Sikorski, Hebert, & Swain, 2008), motor cortex (Ding et al., 2004a; Kleim, Cooper, & Vandenberg, 2002; Swain et al., 2003), and hippocampus of exercising animals (Ekstrand, Hellsten, & Tingstrom, 2008; van der Borght et al., 2009). Further, exercise has been shown to cause the growth of new neurons in the hippocampus, a structure intimately involved in learning and memory (Pereira et al., 2007; van Praag, Kempermann, & Gage, 1999; van Praag, Shubert, Zhao, & Gage, 2005). Interesting, however, is the recent finding that in the hippocampus, the first anatomical response to an exercise regimen is cell death (Kerr & Swain, 2011). This observation suggests that at the start of an exercise program the brain is compromised to such an extent that cells begin to die. One possible explanation for this exercise-induced cell death is that the brain is experiencing a mild form of hypoxia, which would induce the upregulation of HIF-1α and induce several other molecular changes to maintain adequate oxygen supply in the brain.

The purpose of the current study was to determine if exercise is sufficient to induce a mild form of hypoxia in the brain, and therefore the stabilization and upregulation of HIF-1α in the hippocampus of exercising animals. Additionally, the timeline of the HIF-1α expression was assessed and compared to the timeline of exercise-induced angiogenesis. The second experiment was conducted to determine if manipulation of HIF-1α has any effects on exercise-induced angiogenesis. If these two of studies are successful, it is reasonable to conclude that an exercise paradigm can

provide an alternate model with which to study the effects of hypoxia on the brain and can be utilized in the study of stroke and potential therapies to improve the prognosis of stroke victims.

Effects of Exercise on the Brain

In recent decades, the once pervasive belief that changes in brain morphology and vasculature cannot occur into adulthood (Caley & Maxwell, 1970; Rowan & Maxwell, 1981) has been challenged by findings that both neurogenesis (Fabel et al., 2003; Rhodes et al., 2003; Stranahan, Khalil, & Gould, 2006) and angiogenesis (Adkins, Boychuk, Rempel, & Kleim, 2006; Black, Isaacs, Anderson, Alcantara, & Greenough, 1992; Ding et al., 2006a; Isaacs, Anderson, Alcantara, Black, & Greenough, 1992) occur in the brains of adult animals. Angiogenesis, the growth of new capillaries from preexisting vasculature, occurs in pathological conditions, such as the growth of a tumor, and in conditions in which the animal's normal physiological processes are stimulated, such as when an animal is exposed to hypoxia (Harik, Hritz, & LaManna, 1995; Ward et al., 2007) or hypercapnia (Swain et al., 2003). Exercise has also been the topic of several lines of research regarding the effects of physical activity on the vasculature (Adkins et al., 2006; Black et al., 1992; Ding et al., 2006a; Isaacs et al., 1992; Kleim, Cooper, & VandenBerg, 2002; Sikorski, Hebert, & Swain 2008; Swain et al., 2003; Van der Borght et al., 2009), and on the morphology (Ang, Dawe, Wong, Moochhala, & Ng, 2006; Bednarczyk, Aumont, Decary, Bergeron, & Fernandes, 2009; Fabel et al., 2003; Fabel et al., 2009; Kerr & Swain, 2010; Kim et al., 2010; Lou, Liu, Chang, & Chen, 2008; Rhodes et al., 2003) of the adult brain.

Exercise and angiogenesis.

In order for an animal to maintain and improve exercise performance, adaptations in the muscle must occur; not surprisingly, angiogenesis has been found to occur in response to exercise in the skeletal muscle of animals exposed to various exercise regimens. Although not measuring angiogenesis directly, Laufs et al. (2004) identified increases in endothelial progenitor cells in the blood, bone marrow, and spleen of voluntarily exercising mice. Also in this study, human subjects had increases in circulating endothelial progenitor cells, which among other things, function to increase angiogenesis (Laufs et al., 2004). These findings indicate that exercise activates several molecular pathways and processes which are aimed at adjusting the vascular response to the change in activity. The purpose of these molecular changes is to maintain normal peripheral functioning in the wake of exercise and the increase in blood flow that accompanies it.

In conducting a direct assessment of angiogenesis in the periphery of adult animals, Olfert, Howlett, Wagner, and Breen (2010) reported that five weeks of treadmill exercise resulted in significant increases in capillary density in the gastrocnemius muscle in adult rats, and that exercise performance was also significantly increased as measured by maximal running speeds and times to exhaustion throughout the running protocol. Geng et al. (2010) also showed that following endurance exercise, angiogenesis was significantly increased in muscle fibers in mice as measured by the platelet endothelial cell adhesion molecule-1 (PECAM-1). Another study found significant increases in muscle capillarity after 12 days of intensive treadmill running in rats that had undergone

femoral ligation, and these capillaries persisted throughout day 25 when the study ended (Lloyd, Prior, Yeng, & Terjung, 2003).

In addition to exercise-induced angiogenesis in the periphery, angiogenesis also occurs in several regions of the brain in response to exercise (Black et al., 1990; Ding et al., 2006a; Isaacs et al., 1992; Kleim et al., 2002; Sikorski et al., 2008; Swain et al., 2003; Van der Borght et al., 2009). Black and colleagues (1990) found significant increases in capillary density in the cerebella (paramedian lobules - PML) of voluntarily exercising animals as well as animals that were forced to exercise on a treadmill. Interestingly, capillary density in animals learning to navigate acrobatic-type obstacles did not differ from sedentary animals suggesting that aerobic exercise is necessary to produce significant changes in brain vasculature (Black et al., 1990). In a similar study, Issacs and colleagues (1992) also found that animals in both forced and voluntary exercising conditions had significantly decreased diffusion distances in the PML compared to both inactive animals and animals assigned to an acrobatic skill training task. Interestingly, both Black and colleagues and Isaacs and colleagues found that animals trained in the acrobatic condition had significantly greater volume of the molecular layer per Purkinje cell, whereas exercising animals did not demonstrate these morphological changes. These findings are supported by the work of Kleim and colleagues (2002) who reported that the motor maps of the forelimb region of motor cortex in animals given access to a running wheel for 30 days did not change, but significant increases in blood vessel density were identified. Taken together, these findings support the conclusion that repeated motor training is necessary for exercise-induced angiogenesis, whereas skill

learning results in changes in synapses, cell morphology, and movement representations (Black et al., 1990; Isaacs et al., 1992; Kleim et al., 2002).

In addition to being observed in the cerebellum, exercise-induced angiogenesis has been observed in the motor cortex of exercising animals (Ding et al., 2004a; Kleim et al., 2002; Swain et al., 2003). Kleim et al. (2002) found that animals given free access to a running wheel for 30 days had significantly greater capillary density in layer V of the motor cortex compared to inactive animals, and also noted that this change in blood vessel density did not significantly change the animals' motor maps. The change in capillarity in the absence of the change in motor maps is similar to the previously mentioned studies that found increases in capillary density in the PML, without increases in synapses or changes in cellular morphology (Black et al., 1990; Isaacs et al., 1992). The consistency of these findings indicates that simply repeating a movement, such as is done while running, is enough to cause capillaries to grow. Angiogenesis in response to exercise may imply the presence of an adaptive mechanism by which the brain deals with increased blood flow. To this end, Swain et al. (2003) found that voluntary exercise increases the cerebral blood volume in the motor cortex of voluntarily exercising rats, and that this increase in blood volume is observed along with increases in the density of blood vessels within the motor cortex. Changes in the cerebella and motor cortices of exercising animals would be expected given the involvement of these brain regions in locomotion and controlled motor movements (Carlson, 2007). However, exercise-induced angiogenesis in the brain may be a more global phenomenon, affecting several brain regions and not only those associated with motor movement.

As demonstrated by the findings of Swain and colleagues (2003), exercise-induced angiogenesis in the brain occurs with concomitant changes in blood volume and blood flow. Pereira et al. (2007) used an *in vivo* MRI imaging technique to determine if consistent running over a long period of time would induce increases in cerebral blood volume (CBV) in mice. In contrast to Swain et al., these authors focused their study on the hippocampus, an area of the brain known to be involved in learning and memory (Carlson, 2007), and also one of the few regions of the brain to undergo neurogenesis into adulthood (Altman & Das, 1967; Kaplan & Bell, 1984; reviewed in Gould & Gross, 2002). Their findings showed significantly increased CBV in the dentate gyrus of the hippocampus, as well as a trend toward an increase in CBV in the entorhinal cortex in exercising animals (Pereira et al., 2007). Given this increase in blood flow and the motor cortex findings of increased CBV and angiogenesis (Swain et al., 2003), it would be reasonable to conclude that exercise-induced angiogenesis is a process that may be stimulated by one or more processes, such as increased CBV, blood flow, and hypoxia.

Van der Borght et al. (2009) confirmed that exercise does induce angiogenesis in the hippocampus, specifically in the dentate gyrus (DG), by exposing mice to one, three, or ten days of housing with a running wheel. They found that after just three days of running, exercising animals had significantly greater blood vessel density in the DG compared to control animals. This increase in capillarity continued through the ten day time period, and blood vessel density returned to control levels after just one day of housing in standard cages not equipped with wheels. Ekstrand, Hellsten, and Tingstrom (2008) also found significant increases in blood vessel staining in the molecular layer of the hippocampus in voluntarily exercising animals compared to inactive controls,

however similar changes in blood vessel density were not observed in the prefrontal cortices of exercising animals. This finding suggests that exercise-induced angiogenesis may not be a phenomenon that affects the entire brain. The occurrence of angiogenesis in the motor cortex and cerebellum seem to make sense intuitively as these regions of the brain are involved in locomotion. It can also be argued that running on a wheel or treadmill involves some sort of learning, albeit minimal, therefore the hippocampus should also be a brain region that would be expected to undergo exercise-induced angiogenesis. Furthermore, the hippocampus is involved in spatial tasks and configurational mapping (Carlson, 2007), and locomotion by its very nature is a naturally spatial task. As such, the hippocampus is and should continue to be a structure of interest in exercise studies.

The findings that angiogenesis occurs in response to physical exercise has led to research investigating the molecules and molecular pathways involved in mediating exercise-induced vascular plasticity. Research has identified several molecules involved in this plastic process; one of the most important and extensively studied molecules is the vascular endothelial growth factor (VEGF).

Vascular endothelial growth factor (VEGF).

Several normal physiological and pathological angiogenic processes require the activation of VEGF and its successful binding to its high-affinity receptors, the FMS-like tyrosine kinase receptor (Flt-1, also known as VEGFR-1), and the fetal liver kinase 1 receptor (Flk-1, also known as VEGFR-2) (Ferrara, 2004; Risau, 1997). A normal physiological process that requires angiogenesis is the proper functioning and development of the corpus luteum in the ovaries (Benyo, Ravindranath, Bassett,

Hutchison, & Zeleznik, 1993; Ferrara, 2004; Fraser & Wulff, 2003); in turn, this reproductive process also requires VEGF (Ferrara et al., 1998; Ferrara, 2004; Fraser et al., 2000). Additionally, VEGF has been shown to be activated in conditions of hypoxia (Ferrara, 2004; Larrivee & Karsan, 2000; Olfert, Breen, Mathieu-Costello, & Wagner, 2001; Shweiki, Itin, Soffer, & Keshet, 1992), with concomitant increases in blood vessel density (Olfert, Howlett, Wagner, & Breen, 2010; Ward et al., 2007). Pathological conditions such as tumor growth and survival (Folkman, Watson, Ingber, & Hanahan, 1989; Plate, Breier, Weich, & Risau, 1992; Yancopoulos et al., 2000), and wound healing (Brown et al., 1992; Zhang, Oswald, & Lin, 2008) also require the activity of VEGF and its receptors. Finally, VEGF has also been studied in regard to its role in exercise-induced angiogenesis in both the periphery (Ameln et al., 2005; Birot, Koulmann, Peinnequin, & Bigard, 2003; Laufs et al., 2004; Olfert et al., 2001; Olfert et al., 2010; Tang, Xia, Wagner, & Breen, 2010) and the brain (Ding et al., 2006a; Ding et al., 2004b; Sun et al., 2003; Tang et al., 2010).

Various skeletal muscles have been the tissues of interest in most peripheral studies of exercise-induced angiogenesis and VEGF expression (Ameln et al., 2005; Birot et al., 2003; Olfert et al., 2001, 2010). In studies using a single exercise session without pretraining[1], findings have consistently shown increases in both VEGF mRNA and protein and in the number of capillaries surrounding the muscle tissue (Ameln et al., 2005; Gavin, Drew, Kubik, Pofahl, & Hickner, 2007; Gavin et al., 2007b; Gavin, Westerkamp, & Zwetsloot, 2006; Kivela et al., 2008; Olfert et al., 2009). Ameln and colleagues (2005) found that 45 minutes of knee extensions caused an upregulation of

[1] Pretraining, as used throughout this document, refers to a prolonged period of exercise (several weeks in some cases) which is followed by an acute bout of the same type of exercise. For example, rats could be pretrained for eight weeks on a treadmill before an acute bout of treadmill running.

VEGF mRNA in vastus lateralis muscle biopsies in human subjects, with levels peaking 120 minutes following exercise cessation. Additionally, Gavin et al. (2007a) showed that levels of VEGF mRNA and protein were significantly increased in the vastus lateralis muscles of young and aged men after acute maximal knee extension exercises. They also examined the time frame for the increase in VEGF mRNA and protein in the muscle and found that VEGF mRNA expression reached significantly higher levels at two hours post-exercise and was even greater at four hours; VEGF protein was also significantly increased two hours post-exercise but did not continue to increase to the four hour time point. Tang et al. (2010) also identified significant increases in VEGF mRNA and protein in the gastrocnemius muscles of mice exposed to treadmill exercise for just one hour. Finally, Birot and colleagues (2003) exposed animals to intensive treadmill running for 90 minutes and found an overall increase in VEGF mRNA and protein in whole plantaris muscles. Considered together, the results of these studies suggest that a single bout of exercise training can induce significant increases in VEGF mRNA and protein and that pretraining for a prolonged period of time is not necessary to cause changes in the molecular biology of skeletal muscle. Contrastingly, studies utilizing paradigms that include extensive periods of endurance pretraining have yielded significantly different findings with regard to the increases in VEGF expression and muscle angiogenesis.

Using a paradigm that included eight weeks of pretraining followed by an acute bout of exercise, Olfert et al. (2001) reported that treadmill running caused increases in VEGF mRNA in the gastrocnemius muscle of rats, but surprisingly did not cause an increase in the number of capillaries around the muscle fibers. The lack of significant

angiogenesis in response to the one bout of acute exercise could be due to the fact that animals were sacrificed immediately after the acute exercise bout and some research has indicated that it may take capillaries several hours to form in response to exercise (Ameln et al., 2005; Gavin et al., 2007a). Additionally, it is possible that a negative feedback loop affecting the rate of angiogenesis was initiated at some point within the prolonged pre-training period. The degree of capillarity may have reached sufficient levels to maintain continued exercise performance, therefore no differences in muscle capillarity would be observed as all animals had already attained a sufficient amount of capillaries, and addition of more capillaries following the single bout of exercise would provide no further benefit to physiological functioning. This hypothesis was supported by work showing that in humans trained for eight weeks on a knee extension endurance task, the VEGF mRNA response to a single bout of exercise was significantly smaller when compared to the response of subjects who had not been previously trained (Richardson et al., 2000), suggesting that the VEGF and angiogenesis responses to exercise following a significant pre-training period reach a plateau.

More support for the idea of a mechanism by which the exercise-induced VEGF and angiogenic responses are regulated comes from a study conducted by Amaral and colleagues (2008) in which rats were trained for either three days or 13 weeks. In contrast to the findings of Olfert et al. (2001) who failed to find significant differences in muscle capillarity after 8 weeks of training, these authors report that after both three days and 13 weeks of training, exercising animals had significantly greater capillary to muscle fiber ratios compared to sedentary animals. However, they also reported an attenuation of the VEGF response in animals exercising for 13 weeks such that protein levels were

not significantly different than sedentary control animals. These findings are in agreement with Richardson et al. (2000) who noted a significant decrease in the VEGF response in humans who had undergone eight weeks of training. Lloyd and colleagues (2003) also found that just one day of treadmill running induced a significant increase in VEGF mRNA expression in the gastrocnemius and soleus muscles of rats, but this expression declined consistently until expression returned to baseline levels by day 24 of training. Taken together, these data suggest that initially, levels of VEGF mRNA and/or protein increase substantially but then decline as more capillaries are added, after which time VEGF expression is presumably unnecessary to induce further changes to vasculature to maintain optimal exercise performance, adequate blood circulation, and tissue oxygenation.

The previously described research indicates that exercise causes both an upregulation of VEGF and angiogenesis in skeletal muscles, but these studies failed to determine whether VEGF is necessary for exercise-induced angiogenesis to occur. Olfert et al. (2010) confirmed that VEGF is required for exercise-induced angiogenesis in muscle. The authors used a knockout mouse model lacking the myocyte-specific VEGF gene and found that the expression of VEGF in the knockout mice was 95% lower compared to wild-type control animals after six weeks of exercise. Additionally, the capillary density of the gastrocnemius muscle in the knockout mice failed to increase following training, whereas wild type animals showed a dramatic increase in muscle capillarity. Another study conducted by the same group found that myocyte-VEGF deficient mice had significantly decreased levels of VEGF protein and significantly lower capillary density and capillary to fiber ratios compared to wild-type mice following

treadmill exercise (Olfert et al., 2009). These findings suggest that VEGF is an important growth factor involved in exercise-induced angiogenesis in skeletal muscle.

Research has also investigated the role of VEGF in exercise-induced angiogenesis in the brain. Similar to findings in the periphery, studies show that in response to exercise there is an upregulation of VEGF mRNA and VEGF protein which is accompanied by an increase in blood vessel density in the brains of exercising animals (Ding et al., 2006a; Ding et al., 2004a; Tang et al., 2010). Ding et al. (2004a) exposed rats to treadmill running for three weeks and found a significant increase in blood vessel density in the striatum and a trend toward significantly greater blood vessel density in the cortex of exercising rats. Although they did not measure VEGF, other growth factors known to be involved in angiogenesis and neurogenesis, such as brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF) (Ward & LaManna, 2004), were found to be upregulated in response to exercise. In a later study conducted by this group, they did find increases in both VEGF mRNA and protein, along with significant increases in blood vessel density in both the cortex and striatum of aged animals exposed to exercise (Ding et al., 2006a). Finally, Tang and colleagues (2010) also identified increases in VEGF mRNA and protein in the hippocampii of mice following one hour of intense treadmill exercise.

Along with increases in VEGF activity it would follow that the expression of receptors for VEGF would also be increased; without successful binding to its receptors, VEGF would fail to have a significant effect on blood vessel density in response to exercise. As such, several lines of research have addressed possible increases in VEGF receptor expression, focusing mostly on the high-affinity Flk-1 and Flt-1 VEGF receptors

(Bulinski, Thompson, Powell, Sikorski, & Swain, 2000; Thompson, Bulinski, Powell, Sikorski, & Swain, 2000).

The two VEGF receptors have somewhat different functions. The Flk-1 receptor has been shown to be vital in the formation of the vasculature during embryogenesis, as mice lacking this receptor die *in utero* due to a failure to form an adequate vascular supply system (Shalaby et al., 1995; Shibuya, 2006). In mature animals, Flk-1 receptor expression is significantly downregulated compared to its expression in developing embryos (Millauer et al., 1993). Flk-1 is also increased during pathological processes such as tumor growth, and studies have shown that Flk-1 is found in much greater proportion in tumors than in normal vasculature (Plate et al., 1994; Shibuya, 2006). Contrastingly, the Flt-1 receptor is not necessary for the formation of vasculature but rather in the organization of the vasculature (Fong, Rossant, Gertsenstein, & Breitman, 1995). Like the Flk-1 receptor, the Flt-1 receptor is present during embryogenesis but it is also present in large quantities in adult tissues (Peters et al., 1993), indicating that Flt-1 may act to continuously maintain vascular organization (Ferrara & Davis-Smyth, 1997). It has been suggested that Flt-1 serves as a "stop signal" for angiogenesis during embryonic development, which prevents the vasculature from overgrowth and organizes the successfully formed blood vessels into a cohesive network (Fong et al., 1995; Shibuya, 2006). Overall, all of these finding suggest that whereas Flk-1 is more involved in the actual process of the formation of blood vessels, Flt-1 is more important for the organization of the newly formed network of vasculature. As such, increased expression of both Flk-1 and Flt-1 would be expected in response to exercise - Flk-1 to be involved

in the formation of new capillaries and Flt-1 to successfully organize the newly formed vasculature.

As with the expression of VEGF, the expression of the VEGF receptors has been found to be upregulated in both the periphery (Birot et al., 2003; Gavin et al., 2007a; Kivela et al., 2008; Lloyd et al., 2003; Olfert et al., 2001, 2009, 2010), and the brain (Bulinski et al., 2000; Thompson et al., 2000) in response to exercise training. Lloyd et al. (2003) found significant increases in both muscle capillarity and in the expression of VEGF mRNA in skeletal muscles of rats soon after the commencement of a treadmill exercise training regimen. In addition to these findings, they also assessed the expression of Flk-1 and Flt-1 in the muscles and the timelines along which their expressions increased. Their data showed significant increases in both Flt-1 and Flk-1 following just one day of training and declining to near baseline levels around day nine; interestingly, both receptors were upregulated and returned to baseline levels at approximately the same time. These upregulations in VEGF receptors coincided partially with increases in VEGF mRNA (VEGF mRNA remained elevated until day 12), and preceded significant changes in capillary density, which makes sense as the signaling mechanisms needed to be in place before morphological changes to the vasculature could be observed (Lloyd et al., 2003). Along with increases in the expression of VEGF, Olfert et al. (2001) found an increase in the expression of Flt-1 receptor mRNA, but surprisingly not in Flk-1 mRNA in the gastrocnemius muscle of animals that had been exercised for 8 weeks followed by a single exercise bout before sacrifice. As described above, Olfert et al. (2001) also failed to find significant changes in capillary-to-fiber ratio. This failure to identify increases in capillary density corroborates the finding of unchanged Flk-1 mRNA

expression given that Flk-1 activity is involved in the formation of new vasculature. Finally, it could be that the angiogenic response of Flk-1 had been completed at some point during the training and would therefore not be interpreted as having increased by the time of sacrifice. On the other hand, the increased expression of Flt-1 mRNA could reflect the continued need for the organization and maintenance of exercise-induced changes to the vascular system. Furthermore, the increase in Flt-1 mRNA in the absence of increases in capillary to fiber ratio provides further support for the idea that Flt-1 serves as a "stop signal" for continued vessel formation (Fong et al., 1995; Shibuya, 2006).

In contrast to the findings of Olfert et al. (2001), who found an increase in the expression of Flt-1 mRNA, but not Flk-1 mRNA in rats, a study utilizing an acute exercise bout without pretraining found significant increases in Flk-1 mRNA, but not Flt-1 mRNA in young men (Gavin et al., 2007a). As described above, the lack of significant increases in Flk-1 mRNA following prolonged exercise training suggests that the formation of new vasculature may have occurred sometime earlier during the training period, therefore decreasing the necessity for Flk-1 receptors; Flt-1 receptors would still be needed, however, to maintain the newly formed vessels and to inhibit the overgrowth of unnecessary vasculature. In the case of acute exercise however, Flk-1expression would be expected to increase first to promote the growth of the new vessels, whereas the expression of Flt-1 would perhaps be upregulated later when the capillary network would require organization and/or pruning. Thus, as demonstrated by the findings of Olfert et al. (2001) and Gavin and colleagues (2007a), it is possible that long-term training will promote the continued expression of Flt-1 with little or no Flk-1 expression (at the time

of sacrifice, well into the training paradigm), whereas single bouts of exercise will result in increases in the expression of Flk-1 but not significantly change Flt-1 expression. This hypothesis has been partially supported by exercise research in which the tissues of interest were specifically selected regions of the brain (Bulinski et al., 2000; Thompson et al., 2000).

The expression of the Flk-1 and Flt-1 receptors in the cerebella (paramedian lobules) of animals allowed free access to a running wheel for zero, two, four, 10, or 30 days was assessed by immunohistochemistry and it was found that Flk-1 labeling was significantly greater in animals that had exercised for two days compared to all other groups (Thompson et al., 2000). Additionally, Thompson et al. (2000) reported that Flt-1 labeling was significantly decreased at the two day time point. This finding is in agreement with the findings of Gavin et al. (2007a) who reported significant increases in skeletal muscle Flk-1 mRNA expression in men who underwent an acute bout of exercise. Increases in the Flk-1 receptor immunolabeling have also been identified in the motor cortex in response to exercise, but this increase is delayed relative to the increase of Flk-1 labeling in the cerebellum (Bulinski et al., 2000). It should also be noted that the increases in labeling of Flk-1 in the cerebellum and in the motor cortex of exercising rats coincides with increases in capillary density in these regions (Bulinski et al., 2000; Thompson et al., 2000).

VEGF and its high-affinity receptors are not the only molecules involved in the angiogenic response to exercise. Several other growth, or neurotrophic, factors have been identified for their role in exercise-induced angiogenesis in the brain.

Other angiogenic factors and receptors.

The expression of several other growth factors and receptors is increased in response to exercise and may play a role in exercise-induced angiogenesis. Most of these exercise-affected molecules fall into the category of neurotrophic factors (Kandell, Schwartz, & Jessell, 2000), which are substances that promote the growth or survival of neurons by binding to their appropriate receptors on target cells and cell populations and initiate events that result in the growth and stabilization of the cells (Hamburger, 1993; Kandell et al., 2000). The expression of some growth factors and receptors that have been found to be altered in response to exercise include nerve growth factor (NGF) (Ding et al., 2004a), brain-derived neurotrophic factor (BDNF) (Ding et al., 2004a), angiopoietin 1 and 2 (Ang 1, Ang 2) (Ding et al., 2004b, 2006a) and the Ang receptor Tie-2 (Lloyd et al., 2003), peroxisome proliferator-activated receptor γ coactivator 1-α (PGC-1α) (Chinsomboon et al., 2009; Geng et al., 2010), insulin-like growth factor 1 (IGF-1) (Lopez-Lopez, LeRoith, & Torres-Aleman, 2004), and erythropoietin (EPO) (Ameln et al., 2005).

Research has also found increases in NGF in several areas of the brain in response to exercise (Ding et al., 2004a; Neeper, Gomez-Pinilla, Choi, & Cotman, 1996; Zhu et al., 2006). Zhu et al. (2006) found that socially housed animals that were allowed 24-hour access to a running wheel on alternating days for six weeks had significantly greater NGF protein levels in the hippocampus and frontal cortex compared to animals exposed only to social housing. Similarly, in another study increases in NGF mRNA were observed in the hippocampus after two days of wheel running, and in the caudal cortex at two, four, and seven days after wheel running (Neeper et al., 1996). Finally, Ding and colleagues

(2004a) found significantly increased levels of NGF protein in the striatum and cortex of animals exposed to treadmill exercise, a finding that coincided with significant increases in blood vessel density in the striatum. Although NGF is most notably important for neurogenesis, the findings that NGF is activated in response to exercise and that its increased expression coincides with significant angiogenesis, suggest that neurogenesis and angiogenesis are overlapping processes and that in addition to VEGF, NGF may also be involved in exercise-induced angiogenesis. Furthermore, some research has found evidence for a direct relationship between administration of NGF and angiogenesis in the chick embryo (Cantarella et al., 2002), in ischemic hindlimbs of mice (Emanueli et al., 2002), and in rat corneas (Seo, Choi, Park, & Rhee, 2001).

Another notable growth factor that has been consistently shown to increase in the brain in response to exercise is brain derived neurotrophic factor (BDNF) (Berchtold, Costello, & Cotman, 2010; Marais, Stein, & Daniels, 2009; Neeper et al., 1996; Radak et al., 2006; Rasmussen et al., 2009; Zhu et al., 2006). Neeper and colleagues (1996) allowed rats access to a running wheel for two, four, or seven nights and found significant increases in BDNF mRNA in several regions of the brain including the hippocampus, caudal cortex, frontal cortex, and cerebellum. Rasmussen et al. (2009) found that an acute bout of treadmill exercise resulted in significant changes in hippocampal and cortical BDNF mRNA levels in mouse brain, with levels peaking at two hours post-exercise and returning to baseline levels by 24 hours after exercise cessation. BDNF protein has also been found to be increased in the hippocampus of mice that were allowed running wheel access for three weeks and this increase in BDNF protein levels persisted up to two weeks after exercise cessation (Berchtold et al., 2010). Radak et al.

(2006) also noted an increase in BDNF protein following eight weeks of swimming training, but these changes in neurotrophin levels were no longer present after eight weeks of detraining (being sedentary). Finally, Klintsova, Dickson, Yoshida, and Greenough (2004) reported significantly increased levels of BDNF protein in both the cerebellum and motor cortex of animals that had run on a track for seven days, but that these changes in protein expression were no longer significantly different than controls after two weeks of motor activity. Taken together, these findings indicate that voluntary, treadmill, or swimming exercise can cause changes in levels of BDNF mRNA and/or protein. In considering evidence that BDNF serves as an angiogenic factor in some cancers and treatment with an anti-BDNF antibody results in significantly decreased angiogenesis in cancer cell lines (Hu et al., 2007), and given that exercise increases BDNF activity and causes angiogenesis, it is reasonable hypothesize that BDNF may be involved in exercise-induced angiogenesis.

Another family of growth factors known as the angiopoietins (Ang-1 and Ang-2) has been shown to be involved in normal physiological angiogenesis in both developing and mature organisms (Bikfalvi & Bicknell. 2002; Lee, Han, Bai, & Kim 2009) and may be involved in exercise-induced angiogenesis (Ding et al., 2004a, 2006a). Suri and colleagues (1996) found that transgenic mice lacking Ang-1 failed to develop normally and did not live past embryonic day 12. They also found significant abnormalities in the heart vasculature and endothelium of the embryos, indicating that Ang-1 is necessary for functional angiogenesis during development. In addition to its role in developmental angiogenesis, the expression of angiopoietins has also been found to be upregulated following physical activity. Specifically, three weeks of treadmill exercise resulted in

significant increases in Ang-1 and Ang-2 mRNA (Ding et al., 2004b). A similar study conducted by Ding and colleagues (2006a) also found increases in blood vessel density that occurred with concomitant increase in Ang-1 and Ang-2 mRNA, indicating that the angiopoietins may be directly involved in activity-induced angiogenesis.

Exercise and neurogenesis.

Early neuroscience researchers had held to the belief that once an organism reaches maturity or passes some developmental stage, the brain is fixed and unable to alter its morphological or physiological makeup, be it neuron number or vasculature (Caley & Maxwell, 1970; Rowan & Maxwell, 1981). However, more recent studies have investigated the possibility that the brain is not a fixed organ and that it retains the ability to change its anatomy and physiology well into adulthood (Kempermann, Gast, Kronenberg, Yamaguchi, & Gage, 2003; Kempermann, Jessberger, Steiner, and Kronenberg, 2004). Kempermann and colleagues (2004) proposed a systematic series of stages by which newly born hippocampal cells become neurons, survive, and establish connections. Specifically, they describe six stages through which stem cells develop into progenitor cells with certain functional fates and finally become established neurons having created connections with other neurons. Some experiments by Kempermann's group have confirmed this model in mature animals. For instance, they injected adult mice with the cell proliferation marker Brd-U for 12 days and found that one day after the last injection there was a significant increase in newly generated cells in the dentate gyrus (Kempermann et al., 2003). The number of Brd-U positive cells decreased, however, up until four weeks after the last injection at which time the number of new cells remained stable for the duration of the study (11 weeks). These findings indicate that although not

all of the newly formed cells survive to make connections and become mature neurons, cells are continuously being created in the hippocampus of adult animals. Further investigations of neurogenesis in adult animals have focused on behaviorally induced or activity-dependent changes in cell proliferation (Fabel et al., 2003, 2009; Kempermann, Kuhn, & Gage, 1998; Lu et al., 2003; van Praag, Kempermann, & Gage, 1999).

Different housing conditions have been shown to greatly affect the magnitude of neurogenesis in the hippocampus of adult animals. Specifically, research has found that animals housed in group conditions had significantly greater of numbers of immature, newly born neurons (as double-labeled with Brd-U and TOAD-64, a label for immature neurons) in the hippocampus compared to animals housed individually (Lu et al., 2003). Similarly, both young and old mice housed for several weeks in "enriched environments" - where animals had opportunities for increased locomotion, exploration, and interaction with other animals - had significant increases in Brd-U/NeuN-co-labeled cells in the dentate gyrus of the hippocampus compared to animals housed in standard group conditions (Kempermann et al., 1998, p. 3206). However, because enriched environments provide animals with several behavioral options (i.e., physical activity, exploration, and social interaction), it was unclear what aspect of the exposure caused the increases in neuron number. Therefore, van Praag et al. (1999) devised an experiment to determine what components of the enriched environment, if experienced one at a time, would lead to neurogenesis. They found that animals given access to a running wheel were found to have significantly more Brd-U/NeuN-double-labeled cells initially (one day after injection with Brd-U), and these neurons were found to have survived up to four weeks after the last Brd-U injection, indicating that the newly formed cells had matured

and persisted. However, animals housed in enriched environments had significantly greater labeling of mature cells after four weeks, but did not show a significant increase in proliferating cells initially. Moreover, animals performing only Morris Water Maze (MWM) training or participating in a forced swim condition did not show immediate increases in cell number or in the number of surviving cells after four weeks. Thus, the authors concluded that physical activity alone, specifically wheel running, was sufficient to induce increases in both neuron proliferation and survival, whereas exposure to enriched environments resulted only in enhanced cell survival and persistence but not proliferation.

Fabel and colleagues (2009) conducted a study to determine if the effects of exercise and environmental stimulation are cumulative such that animals exposed successively to each condition would have greater enhancement of neurogenesis and cell survival than animals exposed to one condition or the other. Their data indicated that animals exposed to voluntary exercise for ten days followed by 35 days of housing in an enriched environment had significantly more cells colabeled for Brd-U and NeuN (a mature neuronal marker), compared to a group of animals that exercised and were then housed in standard conditions, and to a group of animals that began the experiment in standard housing followed by housing in an enriched environment. These data suggest that continuous exposure to a variety of housing conditions affects neurogenesis to a greater degree than any one treatment alone, thus appearing to yield cumulative changes in neuron proliferation and survival. Furthermore, physical exercise seems to be the driving force behind cell proliferation and that continued activity, even in the form of less intense exercise by way of environmental enrichment, is important for the persistence of

the newly generated cells. Finally, the results of Fabel and colleagues (2009) are in agreement with other studies that showed that environmental enrichment alone is not sufficient to induce cell proliferation (van Praag et al., 1999).

As a departure from research investigating the effects of enriched environments and social housing on cell proliferation and survival, many researchers have focused their attention specifically on the effects of physical exercise, either forced or voluntary, on the proliferation and survival of cells in the hippocampus of adult animals (Bednarczyk et al., 2009; Fabel et al., 2003; Kerr & Swain, 2011; van Praag et al., 1999). Measuring the expression of doublecortin (DCX), a gene that can be found on both precursor cells with a neuronal fate and on immature neurons (Couillard-Despres et al., 2005), Bednarczyk and colleagues (2009) found that mice that were allowed to exercise for six weeks had significantly greater numbers of hippocampal DCX-positive cells compared to inactive control animals, and that DCX expression correlated significantly with the overall distance that the mice ran. Additionally, these authors found that exercising animals had significantly more actively dividing cells, as labeled by Ki67, compared to control mice. Shorter periods of voluntary exercise have also resulted in significant increases in neurogenesis, as demonstrated by Kerr and Swain (2011) who allowed animals free access to a running wheel for six days and found significant increases in DCX labeling in the hippocampus after just 12 hours of running wheel exposure. Furthermore, these significant increases in DCX-positive cells in the hippocampus persisted through day four of wheel running after which DCX levels returned to baseline by the sixth day of running. Taken together, these findings suggest that not only does wheel running result in increases in neuronal precursor cells but also actively dividing cells. Additionally, these

observations indicate that the neurogenic effects of voluntary exercise can be observed using a wide variety of exercise durations, beginning in as little as 12 hours of running exposure and continuing throughout a multi-week training program.

In addition to voluntary wheel running paradigms, forced treadmill exercise has also been found to induce significant changes in neuron number (Kim et al., 2010; Lou et al., 2008; Trejo, Carro, & Torres-Aleman, 2001). A study utilizing a highly intense treadmill exercise regimen (17 m/minute for one hour) found significant increases in Brd-U positive cells with neuronal phenotypes (Trejo et al., 2001). Additionally, using a moderate exercise paradigm designed to compare the differences in hippocampal neurogenesis between young and old exercising rats, Kim et al. (2010) found that although Brd-U positive staining was significantly lower in older exercising rats when compared to young exercising rats, both age groups of rats had increased cell proliferation in response to exercise compared to sedentary control animals of the same age.

In contrast to the findings of Trejo et al. (2001) and Kim et al., (2010) who used high and moderate-intensity treadmill exercise paradigms, respectively, Lou et al. (2008) reported an attenuation of the magnitude of neurogenesis following moderate or high-intensity treadmill exercise paradigms. Specifically, Lou and colleagues investigated whether or not the intensity of treadmill exercise has an effect on the magnitude of exercise-induced morphological changes in the hippocampus. Rats ran on a treadmill at three different intensities varying from low-intensity to high-intensity and interestingly, the authors found that animals that had exercised at the lowest intensity had the greatest neurogenic response. That is, rats exercising at a low intensity had significantly more

Brd-U positive cells, as well as significantly more cells co-labeled with Brd-U and the neuronal marker NeuN in the dentate gyrus compared to inactive animals and moderate- and high-intensity exercisers. The findings of Lou et al. (2008) could indicate the stressful nature of treadmill exercise if the intensity is too great, such that animals exercising at high intensities show neurogenesis levels similar to that of control animals. Nevertheless, treadmill exercise should not be considered a poor paradigm with which to study the effects of physical activity on hippocampal neurogenesis as many studies utilizing varying intensities of exercise have been repeatedly successful in inducing robust neurogenesis (Kim et al., 2010; Trejo et al., 2001). Additionally, it is important to note that for particular investigations, it is necessary that the animals maintain significant levels of activity and forced exercise allows the manipulation of exercise intensity. Voluntary exercise paradigms allow the animal the choice of when and for how long it would like to exercise, whereas forced exercise paradigms ensure that the animal engages in sufficiently intense exercise for a specified duration of time.

Similar to exercise-induced angiogenesis, there are several growth factors that have been identified as either necessary or somehow involved in activity-induced neurogenesis (Fabel et al. 2003; Kim et al., 2010, Lou et al., 2008; Trejo et al., 2001). For example, Fabel and colleagues reported that peripherally produced VEGF is necessary for exercise-induced adult neurogenesis. Surprisingly, the authors found that in response to running, hippocampal VEGF levels were not significantly different than control levels. However, upon injection of a peripheral VEGF antagonist, the neurogenic response in exercising animals was similar to that of non-exercising animals, and was significantly different than exercising mice injected with a control vector (Fabel et al.,

2003). These data indicate that peripherally, but not centrally, produced VEGF is necessary for exercise-induced neurogenesis in the hippocampus of adult animals.

Other growth factors such as IGF-1 (Trejo et al., 2001) and BDNF (Lou et al., 2008) have also been identified as important molecules in promoting exercise-induced neurogenesis. Trejo and colleagues (2001) found that 15 days of intense treadmill exercise resulted in significant increases in IGF-1 in the hippocampii of rats, as well as significant increases in Brd-U positive cells compared to control animals. More importantly, their data illustrate that exercising animals that received infusions of an IGF-1 antiserum failed to experience increases in hippocampal neurogenesis. Similarly, several studies have shown that exercise significantly increases levels of BDNF in the hippocampus of rats (Ding et al., 2004a), and others have reported that along with the increase in BDNF expression, there are significant increases in Brd-U labeling (Farmer et al., 2004; Kim et al., 2010). Furthermore, Rossi and colleagues (2006) showed that knockout mice heterozygous for BDNF failed to show increases in hippocampal neurogenesis in response to housing in an enriched environment; given that the enriched environment includes increases in physical activity, it is likely that BDNF is necessary for activity-induced neurogenesis, but a direct link between BDNF and activity-dependent angiogenesis has not been established.

Along with increases in brain blood vessel and neuron number in response to exercise, the brain undergoes several other significant changes when animals engage in exercise. One such change includes various alterations in synaptic organization and plasticity.

Exercise and synaptic plasticity.

In addition to exercise-induced changes in blood vessel density and in neuron number, changes to the structure and/or chemistry of synapses have also been identified in exercising animals (Bruel-Jungerman et al., 2009; Dietrich, Andrews, and Horvath, 2008; Ding et al., 2002, 2003; Ding, Vaynman, Souda, Whitelegge, & Gomez-Pinilla, 2006; Gomez-Pinilla, Ying, Roy, Molteni, & Edgerton, 2002; Hu, Ying, Gomez-Pinilla, &Frautschy, 2009; Toscano-Silva et al., 2010; van Praag, Kempermann, & Gage, 2000).

Ding and colleagues (2002, 2003) found that in the thalamus of animals that had exercised on a treadmill for 28 days there were identifiable levels of synaptophysin, a protein found in cell membranes that may play a role in the release of neurotransmitter (Kandel et al., 2000), whereas no synaptophysin staining was apparent in the thalamus of sedentary rats. Similarly, mRNA and protein of synapsin I, a molecule that has been implicated in several processes such as axonal changes and the release of neurotransmitters (Purves et al., 2001), was found to be upregulated in the spinal cords of rats in response to both three and seven days of voluntary wheel running (Gomez-Pinilla et al., 2002). Greisbach, Hovda, and Gomez-Pinilla (2009) also demonstrated significant increases in both synapsin I and cyclic-AMP response-element-binding protein (CREB) in the hippocampus of rats following just seven days of voluntary wheel running. Several other synaptic proteins such as glial fibrillary acidic protein (GFAP), synaptophysin, and synaptosome-associated protein-25 (SNAP25) were also found to be significantly upregulated in the hippocampus in response to seven days of voluntary wheel running in rats (Hu et al., 2009). Furthermore, both cytoskeletal and chaperone proteins were found to be upregulated in the hippocampus of voluntarily exercising animals (Ding et al.,

2006a). Of the cytoskeletal proteins, or proteins that are necessary for the structure of the cell and the ability of the cell to change its structure (Purves et al., 2001), Ding and colleagues noted dramatic increases in β-tubulin, α-internexin, and GFAP. They also noted increases in chaperone proteins - proteins that play a role in changing protein shape and conformation (Purves et al., 2001) – such as heat shock protein 8 (HSP8) and heat shock 60-kDa protein, both of which are involved in inhibition of apoptosis and in neuroprotection following injury (Yenari et al., 2005).

Increases in synaptic and cytoskeletal proteins are likely involved in the formation of new synapses, which has also been observed in exercising animals. Dietrich and colleagues (2008) allowed adult mice access to a running wheel for four weeks and found significant increases in the number of neuronal spine synapses in the dentate gyrus and area CA1 of the hippocampus compared to sedentary control animals. Both forced and voluntary exercise also induced the sprouting of mossy fibers in area CA3 of the rat hippocampus, resulting in increased synaptic connections among neurons (Toscano-Silva et al., 2010). Although some studies have identified significant changes in synapse number and fiber sprouting in the hippocampus in response to exercise, other studies that have analyzed different brain structures have failed to identify significant exercise-induced changes in synaptic measures. Black and colleagues (1990) found that voluntary exercise did not result in synaptogenesis in the cerebellum, but motor skill learning did cause significant increases in synapse number. These divergent findings can be explained by several differences in methodology and analysis. For example, synaptogenesis may be more readily identifiable in the hippocampus as neurogenesis occurs on a regular basis in this structure, and even more so in response to exercise.

Thus, it could be that as neuron numbers increase it would follow that the number of synapses would also increase. Because Black and colleagues analyzed the cerebellum, where no neurogenesis occurs, it makes sense that no changes in synapse number were identified. Next, the disparity in findings of increased synapse number could be accounted for by the methods used to quantify synapses. Dietrich and colleagues (2008) used an optical dissector method to quantify the number of hippocampal synapses per cubic micrometer of area, whereas Black et al. (1990) quantified the number of synapses per neuron. Black and colleagues' use of an average of synapses per neuron seems like a more conservative method of synapse quantification, whereas the quantification of total synapses in a given area of tissue is affected by variance in the number of synapses per identified neuron. Although both the hippocampus and cerebellum are known to undergo significant morphological and vascular changes in response to exercise, and although the methods of quantification used by both Black et al. (1990) and Dietrich et al. (2008) are valid, with regard to synapse number either of these methodological differences alone could plausibly account for the opposing results

Changes in synaptic spines and fiber sprouting lead to functional changes as measured by electrophysiological techniques. Specifically, Bruel-Jungerman et al. (2009) observed a significant increase in LTP in the dentate gyri of animals that had exercised for two days. Similarly, O'Callaghan, Ohle, and Kelly (2007) subjected rats to seven days of forced exercise and found significantly increased LTP in the dentate gyrus of the hippocampus compared to control animals. Another study by this group also found that aged animals exposed to a long-term exercise regimen had significant increases in excitatory post-synaptic potential (EPSP) slope compared to same-age sedentary animals,

indicating that exercise alters brain electrophysiology regardless of age (O'Callaghan, Griffin, & Kelly, 2009).

Exercise-induced changes in a variety of molecules that function to alter neurotransmitter release, the structural changes of the neurons themselves, and the increase in signaling (LTP), support the notion that exercise can have significant effects on brain anatomy and morphology, as well as effects on a variety of brain functions. As such, research has continued to investigate the ways that exercise may lead to behavioral alteration, specifically with regard to learning and memory performance.

Exercise and cognition.

In addition to the significant chemical and structural neurological changes that occur following exercise, a great deal of research has investigated the relationship between exercise and cognitive function. In general, results of this vast literature suggest that exercise can lead to significant improvements in cognition in both young and aged people and animals (Chae & Kim, 2009; Erickson et al., 2011; Hillman et al., 2006; Kim et al., 2010; Vaynman, Ying, & Gomez-Pinilla, 2004; Winter et al., 2007). Additionally, the beneficial effect of exercise has been observed not only in neurologically healthy human and non-human animals, but also in those who have sustained neurological damage or suffer from neurodegenerative diseases (Griesbach, Hovda, & Gomez-Pinilla, 2009; Griesbach, Hovda, Molteni, Wu, & Gomez-Pinilla, 2004; Wilson et al., 2002; Winter et al., 2007).

Human studies have found that both acute and chronic exercise regimens can yield improvements in cognitive function (Hillman, Snook, & Jerome, 2003; Hillman et al., 2006; Winter et al., 2007). For example, Winter and colleagues (2007) showed that

following a single intense aerobic workout, adult male participants performed significantly better on a vocabulary learning task compared to performance following either moderately intense exercise or sedentary conditions; reaction time was also significantly faster following the intense exercise condition compared to performance following the other conditions. Hillman et al. (2003) also found that after engaging in a single 30-minute exercise bout, undergraduate students completing a flankers task[2] had significantly increased amplitudes of event related potentials in the brain compared to baseline levels. Additional studies have investigated the effects of exercise on cognition in older participants, and have consistently found that exercising participants perform significantly better than age-matched sedentary subjects on various cognitive tasks. For example, Hawkins, Kramer, and Capaldi (1992) found that older adults who participated in a 10-week aquatic exercise program performed significantly better on attentional tasks following the exercise intervention compared to their baseline pre-exercise performances. Additionally, Hillman and colleagues (2006) found significant decreases in reaction times and increases in response accuracy during a flanker interference task in both young and aged humans who reported the greatest amount of physical activity. These findings indicate that exercise can cause significant changes in behavior, particularly in cognition.

Some more recent studies have utilized brain imaging techniques in order to more fully understand the neurological mechanisms by which exercise changes performance. Prakash and colleagues (2011) found that older and more cardiovascularly fit individuals responded significantly faster and had higher accuracy rates during a modified Stroop

[2] A task in which a participant is shown one of two letters, where responses to each letter differ (for example, left-hand response for letter 'P' vs. right-hand response for letter 'J'). The target letter of a particular trial is then flanked by the opposite letter (i.e., JPJ – a left-handed response would be required). This task was first described by Eriksen and Eriksen (1974).

task compared to less fit older individuals. Furthermore, these authors identified significantly more activation in the "anterior processing regions" of the brain of fit individuals compared to the less fit subjects (Prakash et al., 2011, p. 6). Erickson et al. (2011) also found that the volume of the hippocampus was significantly increased following a one-year exercise intervention in older adults, and improvements in fitness level were positively correlated with spatial memory performance. The findings of these imaging studies suggest that exercise actively changes the structure of the brain, providing neuroscientists with a large scale neurological model of the effects of exercise and how it relates to behavior.

On a more microcellular and molecular level, animal studies have allowed researchers to identify the specific pathways that may contribute to exercise-induced increases in cognition. Uysal and colleagues (2005) exposed young rats to treadmill exercise for five days per week for a period of five weeks and found significant improvements in spatial learning in the Morris Water Maze (MWM). Specifically, compared to control animals, exercising rats took significantly less time to find the hidden platform and spent significantly more time in the correct quadrant during a probe trial. These improvements in cognitive performance coincided with significantly increased neuronal density in areas CA1, CA3, and the dentate gyrus of the hippocampus of exercising animals. Kim et al (2010) also found significantly greater numbers of Brd-U positive neurons in the dentate gyrus of both young and old rats that had engaged in treadmill exercise for 30 minutes per day for six weeks. This change in cell number was accompanied by significantly shorter latency to complete a radial arm maze task compared to age matched sedentary animals.

Other findings have indicated that other processes, such as the exercise-induced increases in brain vascularity are responsible for the cognitive enhancements observed following exercise interventions (Kerr, Steuer, Pochtarev, & Swain, 2010; Rhyu et al., 2010). Rhyu and colleagues (2010) showed that older exercising monkeys (15-17 years of age) had significantly greater vascular volume in the motor cortex compared to age-matched control animals. Additionally, both young and old exercising monkeys performed significantly better in a spatial learning task using the Wisconsin General Testing Apparatus compared to sedentary monkeys. The findings of increased vascularity, albeit in the motor cortex rather than the hippocampus, and the findings of cognitive improvement are consistent with the finding of Kerr et al. (2010), who showed that angiogenesis rather than neurogenesis, is more important for the exercise-induced facilitation of learning in rats. Specifically, Kerr et al. blocked either neurogenesis or angiogenesis by way of AZT or SU5416, respectively, and found that AZT-treated animals performed significantly better during the acquisition trials in the MWM compared to SU5416-treated animals. These findings demonstrate that changes in vasculature are more important for normal MWM acquisition compared to changes in neuron number. Kerr et al.'s findings are supported by research that demonstrated that drug-induced inhibition of neurogenesis does not affect animals' performance in the MWM (Shors et al., 2002). These findings should be pursued further as most research regarding cognition and exercise has focused on the contribution of neurogenesis and neurogenic growth factors rather than angiogenesis as the mechanism by which cognitive improvement operates.

The findings of exercise-induced changes in neuron number coincide with significant increases in the expression of several growth factors and improvements in cognitive performance, suggesting that certain neurotrophins are required for exercise-induced facilitation of learning. BDNF has previously been shown to be increased in response to exercise (Ding et al., 2004a; Farmer et al., 2004; Huang et al., 2006; Lou et al., 2008) and is involved in exercise-induced neurogenesis, therefore it may also be involved cognitive facilitation following physical activity. Kim and colleagues (2010) found that in addition to increased neuron density and improved spatial learning, both young and aged exercising rats had significantly increased levels of BDNF protein in the hippocampus compared to sedentary control animals. Vaynman and colleagues (2004) also found that just one week of voluntary exercise significantly increased levels of BDNF mRNA and significantly increased levels of mRNA for the high-affinity BDNF receptor (TrkB) in the hippocampus; administration of a TrkB antagonist fully ablated the exercise-induced increase in both BDNF and TrkB mRNA. In addition to these molecular changes, these authors found significantly improved MWM performance in exercising animals, and this facilitation of learning was abolished in animals injected with the TrkB receptor antagonist. In another study, this group again found significantly increased levels of BDNF mRNA and significantly better MWM performance in response to one week of voluntary exercise, but also noted significant increases in mRNAs for several metabolic proteins including AMP-activated protein kinase (AMPK), and insulin-like growth factor 1 (IGF-1), and ghrelin (Gomez-Pinilla, Vaynman, & Ying, 2008). Furthermore, blocking the activity of BDNF not only prevented exercise-induced facilitation in the MWM but also decreased the expression of the metabolic proteins,

indicating that BDNF may serve as an important mediator of metabolic function, which may in turn play a role in the behavioral changes stimulated by exercise.

IGF-1 is another growth factor of interest in exercise studies and is necessary for exercise-induced hippocampal neurogenesis (Trejo et al., 2001). Some evidence suggests that IGF-1 mediates the effects of exercise on the expression of BDNF and the concomitant facilitation in learning tasks. Specifically, one study demonstrated that under normal conditions both IGF-1 mRNA and BDNF mRNA levels were increased in response to five days of voluntary exercise, and that in the MWM exercising animals found the hidden platform significantly faster than sedentary animals (Ding, Vaynman, Akhavan, Ying, & Gomez-Pinilla, 2006c). These authors also found that blocking the activity of IGF-1 completely negated memory retention (but not learning acquisition) as determined by the time spent in the correct quadrant during a probe trial. Furthermore, the blockade of IGF-1 blocked the exercise-induced increase in BDNF mRNA in exercising animals, suggesting that IGF-1 is necessary not only for exercise-induced alteration of BDNF expression, but also for certain aspects of exercise-induced cognitive facilitation.

Studies of the effects of exercise on the recovery of cognitive function following traumatic brain injury (TBI) have also provided insight into the molecular mechanisms by which exercise affects cognition. Griesbach et al. (2009) inflicted fluid percussion injuries (FPIs) to rats' brains and provided them with a running wheel for seven days beginning two weeks after injury. After the running period, animals were trained on the MWM and the results indicated that exercising animals that had received FPI found the hidden platform significantly faster than sedentary control rats that had also received FPI.

Moreover, in the hippocampus of both sham-treated and FPI-treated exercising animals, there were significantly higher levels of mature BDNF protein compared to their sedentary counterparts. Levels of phosphorylated synapsin I and phosphorylated CREB were also significantly upregulated in exercising animals with and without injury (Griesbach et al., 2009). Even in conditions of brain injury the powerful effects of exercise on learning behavior and on levels of neurotrophins corroborate the previously discussed findings and suggest that, in particular, BDNF is a mediator of the beneficial effects of exercise on learning performance. Additionally, the findings of learning facilitation along with increases in synaptic proteins and proteins involved in long-term memory (CREB) (Ding et al., 2006a; Gomez-Pinilla et al., 2002; Hu et al., 2009; Kandel et al., 2000) suggest that multiple mechanisms operate together to affect changes in cognitive performance.

Electrophysiological aspects of brain function (i.e., LTP) are also increased in response to exercise (Bruel-Jungerman et al., 2009; O'Callaghan et al., 2007), and have been reported to coincide with improved performance on a variety of learning tasks (O'Callaghan et al., 2009). O'Callaghan and colleagues (2007) showed increased LTP in aged animals that had undergone a long-term exercise program. A later study by this group showed that younger also exhibited enhanced hippocampal LTP after seven days of treadmill exercise (O'Callaghan et al., 2009). Furthermore, the short period of exercise also resulted in significantly facilitated object recognition and MWM performance compared to sedentary control animals. BDNF protein was also found to be significantly upregulated in the dentate gyrus of exercising animals, supporting the hypothesis that

BDNF is a prominent mediator of the effects of exercise on cognition and on electrophysiological brain activity.

The extensive evidence that exercise improves cognitive function in a variety of species and populations within species demonstrates the overarching power of physical activity to change the structure and function of the brain. These findings are broadly applicable across psychological and other scientific disciplines, therefore it is in the interest of researchers to continue to examine the effects of exercise on brain chemistry and function. One relatively new and divisive area of interest in this realm of study is the effect of exercise on processes that are traditionally considered harmful, albeit physiologically normal, such as programmed cell death or apoptosis.

Exercise and apoptosis.

Although exercise has beneficial effects on cognitive function, upregulates several beneficial growth factors, and alters brain morphology in beneficial ways, exercise can also have potentially damaging effects by way of the activation of some destructive processes such as free radical formation and cell death (Aguiar et al., 2008; Carraro & Franceschi, 1997). In particular, the occurrence of apoptosis or programmed cell death and its relationship to exercise has been studied fairly extensively in both the periphery and in the brain (Carraro & Franceschi, 1997; Kim et al., 2002; Kim et al., 2010; Phaneuf & Leeuwenburgh, 2000; Uysal et al., 2005). However, there is somewhat of a debate in the literature about the effects of exercise on apoptosis in the hippocampus. Some research suggests that exercise either suppresses or does not alter apoptotic activity in exercising animals (Chae & Kim, 2009; Kim et al., 2002; Kim et al., 2010; Uysal et al.,

2005), whereas other findings have found that exercise causes increases in apoptotic signals (Kerr & Swain, 2011).

Apoptosis is a normal process that can be initiated by way of injury or normal physiological processes such as aging (Pollock, Phaneuf, Dirks, & Leeuwenburgh, 2002) and during exercise (Phaneuf & Leeuwenburgh, 2001; Wyllie, 1997). In mammals, some effectors of apoptosis are the caspases, a family of enzymes, which are suppressed by various other molecules (i.e., bcl-2) in the absence of significant injury or physiological stimuli (Wyllie, 1997). As such, most measures of apoptotic activity focus on the presence of the caspases or on the activity of the suppressor molecules.

In a recent study, Kerr and Swain (2011) found support for the claim that exercise causes apoptosis. Using a range of time points from 12 hours to six days, animals were allowed to voluntarily exercise and levels of caspase-3 were quantified in the hippocampus and cerebellum. Their results showed that caspase-3 began to increase after only 12 hours of wheel running. After 24 hours of exposure to voluntary exercise, levels of caspase-3 activity were significantly higher compared to control animals, and expression returned to control levels by the 48 hour time point. These findings suggest that exercise may be placing sufficient physiological strain on the brain such that cells begin to die. More specifically, the authors claim that it is possible that increases in energy demand during exercise may lead to the death of some cells. The finding of significantly increased apoptotic signals shortly after the initiation of exercise, but not at later time points, also suggests that the apoptotic response to exercise occurs relatively quickly after the commencement of an exercise regimen. Specifically, it is possible that apoptotic signals decreased significantly at the later time points because by that time, the

brain may have adapted physiologically to the exercise-induced increased energy demand and in turn protected the cells from death. There are a multitude of mechanisms by which these changes may have taken place, one of which could be an increase in HIF-1α. This assumption is partially supported by the additional finding of Kerr and Swain (2011) that peak levels of angiogenesis were found after the peak in apoptosis as HIF-1α activation results in increased activity of VEGF, a known mediator of exercise-induced angiogenesis (Bulinski et al., 2000; Thompson et al., 2000).

In contrast to the findings of Kerr and Swain (2011), Kim and colleagues (2002) failed to find significantly increased apoptotic signals in the hippocampus of animals exposed to treadmill exercise. After seven days of running at either easy or moderate levels, these authors found comparable numbers of TUNEL-positive cells in all groups (control, easy exercise, and moderate exercise). These findings provide evidence against the increase in apoptosis in the hippocampus of exercising animals, but a few methodological issues should be taken into account when comparing these findings to the results of Kerr and Swain's (2011) study. First, the qualitative difference in exercise paradigms – treadmill (forced) versus wheel running (voluntary) exercise - may be enough to account for the differences in quantification of apoptosis. Additionally, Kerr and Swain did not identify significant increases in apoptosis until 24 hours after the onset of exercise, whereas Kim and colleagues sacrificed animals just two hours after exercise cessation which might not have been enough time for the apoptotic cascades to affect actual cell death. Furthermore, Kerr and Swain (2011) found that apoptotic signals returned to normal levels at the 48-hour time point. It is possible that because Kim and colleagues allowed animals to exercise for seven consecutive days, animals may have

adapted physiologically to the increased energy demand. As such, levels of apoptotic signals would not be significantly higher because the brain would have already adapted to the increased energy demand at some point earlier in the seven day period. Although these significant methodological differences raise questions about whether exercise does in fact induce apoptosis, other studies have found support for the idea that apoptosis is suppressed rather than stimulated via exercise.

Uysal et al. (2005) investigated the effects of exercise on apoptosis in young rats following a long-term exercise protocol (eight weeks) and failed to find significant differences in apoptosis between exercising and sedentary animals. Another study yielded similar results in both young and aged animals that were exposed to six weeks of exercise during which they ran every day for 30 minutes (Kim et al., 2010). Specifically, Kim and colleagues found that of the exercising animals, both age groups had significantly decreased caspase-3 labeling than their age matched sedentary cohorts. To demonstrate that aged animals have greater baseline levels of apoptosis, they also reported that aged control animals had significantly greater numbers of apoptotic cells compared to young control animals as measured by caspase-3 immunohistochemistry. Finally, Chae and Kim (2009) artificially induced aging by injecting young animals with D-Galactose (an agent that stimulates oxidative stress and cell death; Zhang, Li, Cui, & Zuo, 2005) in young animals and measured apoptotic activity in both exercising and control animals. Exercising animals were exposed to treadmill running for five days per week for five weeks, and the intensity of exercise was gradually increased week-by-week for the duration of the training. Chae and Kim confirmed that the induction of aging resulted in significantly greater levels of apoptotic activity in sedentary aging and

sedentary control (young) animals, and also found that the aging sedentary animals had the greatest number of TUNEL-positive staining and caspase-3 activity compared to both young control and aging-induced exercising animals. All of these studies suggest that exercise, specifically forced or treadmill exercise, results in the suppression of apoptosis, no matter the age of the animal. Furthermore, the suppression of apoptosis in populations in which significant increases in cell death are normally observed suggests that exercise provides significant anti-apoptotic functions. Evidence in support of this assumption is demonstrated in investigations of the effects of cerebral ischemia on apoptosis.

It has been well-established that exposure to an exercise regimen prior to the onset of stroke can result in significantly better recovery as evidenced by decreased infarct volume and neurological deficits, and these outcomes occur concomitantly with significantly greater levels of neuroprotective growth factors (i.e., BDNF, VEGF, etc.) (Ding et al., 2004a, 2004b). Similarly, some work has shown that exposure to exercise shortly after an infarct can be beneficial. Sim and colleagues (2005) found that exposure to four weeks of exercise beginning on the day after the induction of common carotid artery occlusion (CCAO – an animal model of ischemia) and reperfusion significantly reduced ischemia-induced apoptosis in the hippocampus of exercising animals compared to sedentary controls. This work provides additional evidence that exercise serves some sort of anti-apoptotic functions, even in at-risk populations. Despite the inconsistencies in defining the relationship between apoptosis and exercise, it may be that long-term exercise regimens yield significant suppression of apoptosis (Chae & Kim, 2009; Kim et al., 2002; Kim et al., 2010; Sim et al., 2005; Uysal et al., 2005), whereas short-term, voluntary exercise results in significant increase in apoptosis (Kerr & Swain,

2011). Therefore, these results are difficult to compare. For example, it is possible that the prolonged forced exercise protocols caused significant physiological adaptations during the extended periods of exercise training. Therefore, at some point the cells would no longer be compromised in terms of oxygen and metabolic needs, decreasing the likelihood of cell death. In contrast, if it is presumed that apoptosis is suppressed by way of significant increases in growth factors and downstream vascular and morphological changes, then exposure to a shorter-term exercise program may not allow enough time for the induction of these anti-apoptotic physiological changes. Despite the differences in the type of exercise – voluntary versus forced – it is reasonable to hypothesize that a short term, moderately intense exercise paradigm will induce increased apoptosis simply because the animal is not sufficiently physiologically prepared or conditioned to the increases in oxygen and energy demand. As such, it is possible that at the beginning of an exercise program the brain experiences a mild state of hypoxia which would stimulate significant changes in brain chemistry and in turn physiology and morphology.

Effects of Hypoxia on the Brain

Several lines of research have investigated the effects of hypoxia on the central nervous system, focusing on the brain's ability to adapt to conditions of low oxygen tension (Boreo, Ascher, Arregui, Rovainen, & Woolsey, 1999; Chavez, Agani, Pichiule, & LaManna, 2000; LaManna, Chavez, & Pichiule, 2004). Any oxygen level below approximately 21% oxygen is considered to be hypoxic (Lindeburg, 2006), and research varies with regard to the severity of experimental hypoxic exposures. Studies looking at the effects of hypoxia in vitro can use severely oxygen-limited environments, whereas in studies using animals oxygen levels are varied such that the animals survive and

ultimately adjust to the hypoxic environment via various morphological and metabolic alterations in brain structure and brain chemistry (Boreo et al., 1999; Chavez et al., 2000; Harik et al., 1995; LaManna et al., 2004; Mironov, Hritz, LaManna, Hudetz, & Harik, 1994).

Changes in brain oxygenation provide the impetus for the downstream changes in brain structure and chemistry. Upon chronic exposure to low pressure environments and therefore lower oxygen environments, changes have been observed in brain oxygenation. For example, Dunn and colleagues (2000) used electron paramagnetic resonance oximetry to measure the oxygenation of the brains of animals being acclimated to hypobarically simulated high altitude (low pressure and low oxygen conditions). Their results showed that over the course of 30 days, the brain oxygenation of animals placed in the hypobaric chambers was significantly greater than animals housed in normobaric chambers. Specifically, the adaptation of brain oxygenation was rapid, with significant increases observed as early as day four with continued increases until day seven, when brain oxygenation plateaued and remained consistently higher for the remainder of the 30 day study. These data and the findings of other studies that have shown increases in cerebral oxygenation and blood flow in response to simulated hypoxic conditions (Yang, Bergo, Krasney, & Krasney, 1994; LaManna, Vendel, & Farrell, 1992) indicate that basic processes such as changes in blood flow, and therefore oxygenation levels, begin the cascade that ultimately culminates in major changes to brain metabolism and vasculature.

LaManna et al. (1992) found significant increases in cerebral blood flow in adult rats that had been exposed to 15 minutes of hypoxia, and these significant increases were maintained in animals that were exposed to hypoxia for three hours. Similarly, tissue

oxygenation in the brain as measured by oximetry (a method developed to measure the partial pressure of oxygen in brain tissue) has been reported to be increased significantly in animals exposed to long bouts of hypoxia (Dunn et al., 2000). In addition to significantly increased cerebral blood flow and oxygenation in rats exposed to hypoxia, metabolic changes involving significant increases in glycolytic pathway molecules – such as pyruvate, lactate, and glucose 6-phosphate – have been observed (Beck & Krieglstein, 1987). Glucose transporter 1 (GLUT-1), an important mediator of energy metabolism (Olson & Pessin, 1996) was also increased in capillaries of rats exposed to one or three weeks of hypoxia, and vessel GLUT-1 levels remained elevated even after three weeks of housing in normoxic conditions (Harik et al., 1996). These results suggest that in response to hypoxia, the brain's initiation of adaptive processes begins with an increase in blood flow and changes in metabolism which, if hypoxic conditions are prolonged for a significant period of time, leads to other changes such as restructuring of the microvasculature of the brain.

In an effort to maintain sufficient oxygenation in the brain in conditions of hypoxia, restructuring of the vascular network in the brain is necessary (LaManna et al., 2004). To that end, researchers have identified significant increases in capillary density, and decreases in inter-capillary distancing (Boreo et al., 1999; Harik et al., 1995; Lauro & LaManna, 1997). Boreo and colleagues (1999) found increases in capillary length and diameter, and decreases in the distance between capillaries in several regions of the brain including the hippocampus of mice following four weeks of housing in a hypobaric chamber.

Similarly, LaManna and colleagues (2004) identified significant increases in blood vessel density in several brain structures including areas CA1 and CA3 of the hippocampus in animals that had been exposed to three weeks of hypoxia. Shorter term hypoxic exposure of just one week caused significant increases in microvessel protein measurements from the brains of rats, and rats housed for either two or three weeks in hypoxic conditions had significantly increased measures of brain microvessel DNA suggesting significant increases in vessel density (Harik et al., 1995). Another study by Harik and colleagues (1996) showed significant increases in the vascularity of the motor cortex of rats following both two and three weeks of hypoxic exposure. Furthermore, after a three week recovery period of housing in normoxic conditions, capillary density returned to control (normoxic) levels. This return to control levels is similar to findings regarding cerebral blood flow. LaManna and colleagues (1992) showed that after three weeks of hypoxic exposure followed by four hours of normoxic recovery, blood flow levels did not differ between hypoxia-exposed and control rats.

These findings highlight the readiness with which the brain adapts to changes in oxygen tension, from either high to low oxygen levels or low to high oxygen levels, and suggests that if conditions of altered oxygen availability are present, animals physiologically adjust to maintain adequate function. Most notably, the findings that as little as 15 minutes of hypoxic exposure can induce significant changes in cerebral blood flow (LaManna et al., 1992), and the finding that blood flow returns to normal levels within just four hours after a prolonged housing in hypoxic conditions (Harik et al., 1996) suggest that the brain has the ability to adapt its physiology relatively quickly. As such,

other conditions that create a hypoxic environment in the brain such as ischemia or stroke may result in rapid and significant changes to brain chemistry and physiology.

Ischemia causes a myriad of changes in the brain. When blood flow is disrupted, the affected region of the brain lacks an adequate supply of oxygen and therefore cannot continue to function normally (Fawcett et al., 2001). This lack of blood flow and the resulting hypoxia lead to several damaging processes including excessive glutamate release and NMDA and AMPA receptor activation (also known as excitotoxicity), formation of free radicals, and depending on the severity of the insult, cell death (Fawcett et al., 2001; Won, Kim, & Gwag, 2002).

One of the brain's responses to ischemia is excitotoxicity which is caused by a series of events that can ultimately result in cell death. The general mechanism of this cell damage begins with the excess release of glutamate (Takagi et al., 1993; also reviewed in Choi, 1992). The overabundance of glutamate results in changes in the concentration gradients of ions across the cells, ultimately resulting in the diffusion of sodium (Na^{+}) and chloride (Cl^{-}) into the cell which causes the cell to swell and possibly die (Choi, 1992; Won et al., 2002). In addition to alteration of the balance of sodium and chloride inside and outside of cells in the ischemic area, the intracellular concentration of calcium (Ca^{++}) also increases and is lethal to the cell if the levels become too high (Benveniste, Jorgensen, Diemer, & Hansen, 1988; Choi, 1992). The interrupted balances of ions inside and outside of cells are affected mainly through excessive activation of NMDA receptors. Briefly, loss of blood flow or oxygen to cells results in glutamate release which leads to cell depolarization. This depolarization then causes activation of NMDA receptors resulting in the influx of large amounts of calcium. The extra supply of

glutamate propagates these responses until the excess levels of Ca^{++} reach high enough levels to cause significant neuronal injury or death (Fawcett et al., 2001; Szatkowski & Atwell, 1994; Won et al., 2002). The importance of NMDA receptors and their calcium binding properties during hypoxic or ischemic events has been demonstrated by several studies that have shown that antagonizing NMDA receptors upon initiation of the ischemic event decreases infarct size and cell death (Dawson, Graham, McCulloch, & Macrae, 1994; Fujisawa et al., 1993).

To counteract the deleterious effects of an ischemic event, other processes are set in motion that are meant to assist the brain in returning to a state of metabolic homeostasis, and to potentially salvage cells from further damage. After initiation of ischemia, one of the major changes is in blood flow. Takagi et al. (1993) subjected rats to two hours of middle cerebral artery occlusion (MCAO) and found that cerebral blood flow decreased significantly immediately after ischemia onset, but that blood flow continued to increase slightly throughout the remainder of the ischemic period. Upon removal of occlusion, blood flow increased dramatically to greater than 100 percent of baseline flow values (Takagi et al., 1993). These findings mirror the findings of studies that have exposed animals to hypoxic conditions without induction of ischemia, where cerebral blood flow and brain oxygenation has been shown to increase shortly after the decrease in oxygen availability (Dunn et al., 2000; LaManna et al., 1992).

Angiogenesis has also been shown to occur following ischemia. Krupinski, Kaluza, Kumar, Kumar, and Wang (1994) showed that cerebral capillary density was significantly higher in the ipsilateral hemisphere compared to the contralateral hemisphere of human stroke patients who had survived for up to three months post-

ischemia, and that the increase in vessel density was significantly positively correlated with survival time. Using an animal model of stroke, Zhang et al. (2002) found that two weeks after stroke, there was a significant increase in small diameter vessels compared to similar regions in the opposite hemisphere. Similarly, another study noted significant changes in vessel characteristics in the area surrounding an infarct induced by MCA ligation in rats (Wei, Erinjeri, Rovainen, & Woolsey, 2001). Specifically, these authors found that one month after stroke vessels were significantly remodeled and staining for the angiogenic integrin $\alpha_V\beta_3$ was particularly evident in at the border of the infarct at ten days post-surgery (Wei et al., 2001).

As would be expected with the occurrence of angiogenesis, VEGF has also been found to be significantly increased very shortly after ischemia. Zhang et al. (2002) identified significant increases in VEGF mRNA expression in rat brain just two to four hours after onset of stroke. In addition, expression of the VEGF receptors flk-1 and flt-1 were also significantly increased from two days to four weeks after stroke. Hayashi, Noshita, Sugawara, and Chan (2003) measured gene expression following cerebral ischemia in mice and found significant increases in VEGF gene expression after just one hour of blood reperfusion and levels of gene expression remained elevated at one day following reperfusion, but decreased to baseline levels at the three week time point. In addition, they also found significant increases in the expression of the gene for the VEGF receptor, flk-1, after just one hour of reperfusion, and levels of this receptor remained elevated throughout the 21 days of the study. Finally, Hayashi and colleagues identified significant angiogenesis beginning at the three days after ischemia, and angiogenesis continued to increase through days seven and 21. Another study by this group also found

significant elevations in VEGF mRNA and protein expression just one hour after reperfusion following MCAO in rats (Hayashi, Abe, Suzuki, & Itoyama, 1997). These findings suggest that very soon after the initiation of ischemia, angiogenic processes are set into motion with increased expressions of VEGF and its receptor. Presumably, this fast response to ischemic conditions occurs in an effort to maintain or return the brain to adequate oxygenation conditions, and this idea is supported by findings that report that application of VEGF after an ischemic insult can rescue cells from damage and ultimately decrease the size of the infarct (Hayashi, Abe, & Itoyama, 1998).

Although protective mechanisms are in place to try to assuage excess damage to cells upon induction of hypoxia or ischemia, there is some evidence that different regions of the brain are more susceptible to hypoxic or ischemic insults (Arai, Passonneau, & Lust, 1986; Bonnekoh, Barbier, Oschlies, & Hossmann, 1990; Kreisman, Soliman, & Gozal, 2000). Specifically, within the hippocampus, area CA1 is thought to be much more vulnerable to decreased oxygenation compared to area CA3. Kreisman and LaManna (1999) reported that upon bathing rat hippocampal slices in a solution of 95% N_2 and 5% CO_2, neurons in area CA1 of the hippocampus became swollen (as measured by a significant decrease in the transmission of light through that region), and the rate of neural depolarizations increased significantly when compared to exposure to a medium of 95% O_2 and 5% CO_2. These responses are similar to those observed in excitotoxicity as described above: cell swelling, presumably caused by increases in intracellular Na+ concentration, and depolarization caused by an excessive release of glutamate (Szatkowski & Atwell, 1994; Won et al., 2002). In a follow-up study to determine if responses were regionally different throughout the hippocampus, Kreisman et al. (2000)

evaluated the activity of neurons in the dentate gyrus (DG), CA1, CA3 in response to incubation in a solution of 95% N_2 and 5% CO_2. Their findings showed that CA1 neurons swelled and depolarized to a significantly greater extent than neurons in either CA3 or in the DG, suggesting that CA1 neurons are especially vulnerable to these types on insults.

Like hypoxia, induction of ischemia has also been shown to induce preferential damage in area CA1 of the hippocampus. Arai and colleague (1986) used a gerbil model of ischemia and reported that at four days after subjecting animals to bilateral common carotid artery occlusion, almost the entirety of CA1 neurons had died, but CA3 neurons appeared to be mostly unaffected by ischemia. These authors did not quantify neuron number, but their observations have been confirmed by similar studies. For example, bilateral occlusion of the common carotid arteries in gerbils for just five minutes resulted in significantly decreased neuron numbers in area CA1 of the hippocampus compared to control animals three weeks after ischemia (Bonnekoh et al.,1990). Additionally, there was no difference in neuron number in the DG or area CA3 between ischemic and control animals. Furthermore, these authors also reported that while neurogenesis occurred in animals that had undergone ischemia, there remained a significant difference in neuron number in CA1 between control and ischemic animals at both six and ten weeks post-ischemia (Bonnekoh et al., 1990).

Other brain injuries have also been shown to more substantially affect area CA1 compared to CA3. Griesemer and Mautes (2007) induced a brain injury in young rats and found that neurons in area CA1 produced significantly more non-evoked action potentials compared to sham animals, however, this “hyperexcitability” was not found in

CA3 neurons after injury (Griesemer & Mautes, 2007, p. 1827). Based on these findings, it appears as if brain injury induces regionally-specific changes in the hippocampus similar to those observed in conditions of hypoxia or ischemia, namely the increased excitability of cells in CA1 but not in CA3 (Greisemer & Mautes, 2007; Kreisman et al., 2000). However, it should be noted that some aspects of the effects of brain injury as opposed to a hypoxic or ischemic episode, and their effects on specific regions of hippocampus are different. Although Greisemer and Mautes failed to find significant changes in excitability of CA3 neurons following brain injury, they observed (but did not statistically analyze) a clear difference between the two regions in the amount of intact neurons following injury, with degeneration of neurons appearing to be much more abundant in CA3 neurons compared to CA1 neurons. Therefore, although some aspects of hippocampal damage following hypoxia or ischemia and brain injury seem to be similar, there is still the possibility of significant differences in findings between the two injury paradigms.

The differences between responses to changes in oxygenation between areas CA1 and CA3 of the hippocampus should continue to be investigated. Based on the findings described above, namely that CA1 is more susceptible to injury following hypoxia, it is possible that regional differences in morphology and neurochemistry in response to exercise would also be observed. In the context of the present study, regional differences in the expression of hypoxia-inducible molecules will be examined to determine if the brain's response to exercise is similar to that of hypoxic or ischemic conditions. If exercise and hypoxia activate similar pathways, and given that area CA1 of the hippocampus is more vulnerable to decreased oxygen, we would expect to see greater

expression of hypoxia-sensitive molecules in this area when compared to CA3 which is not as easily damaged following hypoxic insult.

Hypoxia-inducible Factors (HIFs)

The HIFs are transcription factors that belong to the basic helix-loop-helix (bHLH) family of transcription factors (Chen, Endler, & Shibasaki, 2009). Helix-loop-helix (HLH) proteins are required for a variety of functions throughout the life of an organism, from development to sex determination to neurogenesis (Murre et al., 1994). They function as regulatory proteins that contain specific regions for DNA binding and for dimerization between different subunits of other proteins (Lewin, 2008; Murre et al., 1994), and most importantly in the present context, allow for the regulation of gene transcription via binding response elements, for example the hypoxia response elements (HREs) (Lewin, 2008; Murre et al., 1994). Some HLH proteins are basic (bHLH), whereas other HLH proteins are nonbasic; the basic region is required for DNA binding, and the helical regions are needed for dimerization of subunits or other proteins (Lewin, 2008). As bHLH proteins, HIFs dimerize, bind to HREs, and activate the transcription of several genes including the growth factor VEGF, and the hormone EPO (Harten et al., 2010).

HIFs consist of two subunits, an alpha (α) subunit and a beta (β) subunit, which must dimerize in order to exert their effects on cells in various physiological and pathological conditions (Acker & Acker, 2004). The α subunit of the HIFs is the oxygen-sensitive subunit, whereas the β subunit is transcribed continuously and is not sensitive to oxygen levels (Chen et al., 2009; Jewell et al., 2001; Stroka et al., 2001). Under conditions of normal oxygen tension, or ~21% O_2, the α subunit is rendered ineffective

via a pathway of enzyme degradation. Specifically, a group of enzymes known as prolyl-4-hyroxylases (PHDs) bind to oxygen-sensing prolines on the HIF-α subunit, which allow for the binding of a complex of degradation enzymes containing the von Hippel Lindau protein (VHL). Binding of the VHL complex to the hydroxylated prolines of HIF-α leads to the ubiquitination and subsequent proteasomal degradation of the subunit (Chen et al., 2009; Harten, Ashcroft, & Maxwell, 2010; Sharp & Bernaudin, 2004). In conditions of hypoxia, the proline residues on the α subunit are not hydroxylated and are therefore not targeted by the VHL complex for degradation; the subunit is not broken down but stabilized and allowed to accumulate (Harten et al., 2010; Sharp & Bernaudin, 2004). Upon accumulation, the α subunit dimerizes with the β subunit and translocates to the nucleus, where it binds with the hypoxia response elements of several genes including those that encode vascular endothelial growth factor (VEGF), erythropoietin (EPO), and glucose transporter 1 (GLUT-1), just to name a few (Harten et al., 2010; Sharp & Bernaudin, 2004).

There are three HIF isoforms: HIF-1, HIF-2, and HIF-3. The α subunits of the HIF-1 and HIF-2 isoforms have similar functions and are both induced in hypoxia and cancer, among other pathological conditions (Chen et al., 2009; Ema et al., 1997; Harten et al., 2010; Sharp & Bernaudin, 2004; Tian, McKnight, & Russell, 1997). Furthermore, HIF-1α and HIF-2α (also known as endothelial PAS-domain protein 1 – EPAS-1) both have hypoxia response elements (HREs) with which target genes such as VEGF and EPO interact (Ema et al., 1997; Tian et al., 1997). Contrastingly, Makino et al. (2001) showed that HIF-3α, commonly known as inhibitory PAS domain protein (IPAS), when co-expressed with HIF-1α and HIF-2α in tissues under hypoxic conditions, functions to

negatively regulate the gene expression induced by the other two isoforms. Therefore, HIF-1α and HIF-2α are the isoforms of interest in hypoxia, and HIF-3 α is generally not considered as important in terms of the hypoxic induction and downstream regulation of gene transcription (Chen et al., 2009; Ema et al., 1997; Pugh & Ratcliffe, 2003; Tian et al., 1997).

HIF-1α versus HIF-2α.

As both HIF-1α and HIF-2α have HREs present on their promoter regions (Ema et al., 1997; Fong, 2009; Tian et al., 1997), both have been found to induce several genes important for stimulation of the angiogenic process following exposure to hypoxic conditions. Tian et al. (1997) isolated the cDNA for the coding region of mouse HIF-2α (EPAS-1) and showed that the sequence was very similar to the sequence of HIF-1α. Additionally, Tian and colleagues sought to determine if the two HIF-α isoforms were selectively expressed in different tissue types. They showed that HIF-1α and HIF-2α are both highly expressed in almost all tissue types in embryonic samples, and low levels of both isoforms were also identified in all tissue samples from adult mice (Tian et al., 1997). Additional studies have demonstrated differential expression for the HIF-1α and HIF-2α, based on tissue type and the specific conditions to which cells or animals are exposed.

To determine which HIF-α isoform is most important in cancer-induced hypoxic regulation of genes, Sowter, Raval, Moore, Ratcliffe, and Harris (2003) used siRNA to silence the expression of each isoform in different types of peripheral cancer cells *in vitro*. Sowter and colleagues showed that upon exposure to hypoxia, two lines of breast carcinoma cells showed an upregulation of genes important for the maintenance of

homeostasis in response to hypoxia, including VEGF and GLUT-1. Results also indicated that in those cells positive for both HIF-1α and HIF-2α, blocking HIF-1α but not HIF-2α, resulted in inhibition of the induction of the hypoxia-responsive genes examined. This indicates that under hypoxic conditions, HIF-1α may be more important in the transcription and regulation of homeostasis-maintaining molecules such as VEGF and GLUT-1 (Sowter et al., 2003), and is in agreement with the findings of O'Rourke, Tian, Ratcliffe, and Pugh (1999), who reported that HIF-1α is more substantially upregulated in conditions of hypoxia, whereas HIF-2α is more highly active in normoxic conditions; Weisener et al. (1998) also found that HIF-2α protein was found in both normoxic and hypoxic cells.

However, there is some evidence that suggests HIF-2α is more important in regulating oxygen homeostasis by way of the VEGF response. Rankin and colleagues (2008) developed a line of mutant mice in which HIF-2α was inactivated. They found that both HIF-1α and HIF-2α were present in mice that developed hepatic tumors, but that blocking HIF-1α did not change the appearance of the tumors. Furthermore, mutant mice with inactivated HIF-2α only, resulted in a decrease in VEGF activity in the tumor. In contrast to Sowter et al.'s (2003) findings, Rankin et al. (2008) concluded that HIF-2α was the dominant isoform in the management of vascularization. It should be noted, however, that different peripheral cell types were used in the two studies, suggesting that each isoform may have very specific patterns of expression, and the patterns may be dependent on the cell types involved. This observation is supported by the finding that HIF-1α protein expression tended to be somewhat restricted to the regions just outside of necrotic areas in breast adenocarcinoma tumors (Talks et al., 2000); these findings also

suggest that HIF-1α may be more active in regions near an infarct or tumor that are still salvageable. Another possible explanation for the discrepancy of expression of the different isoforms among different cell types is that new vascularization and angiogenesis are fundamentally different processes (Larrivee & Karsan, 2000), therefore it may be that for neovascularization HIF-2α is the predominantly active isoform, but for the sprouting of new capillaries from a pre-existing network in response to hypoxic conditions, HIF-1α is the greater contributor.

Regarding the differential expression of the two HIF-α isoforms, there is evidence that levels of HIF-1α and HIF-2α protein are preferentially expressed to a greater or lesser degree depending on tissue type. Sowter et al. (2003), demonstrated that both HIF-1α and HIF-2α protein were present in one of two lines of breast carcinoma cells and in human umbilical vein endothelial cells (HUVECs), but only HIF-2α RNA was expressed in renal carcinoma cells. Interestingly, Weisener et al. (1998) exposed cancer cells originating from 11 different peripheral tissues to normoxia and hypoxia, and found that in most of the cell lines examined, HIF-2α mRNA was expressed in a much larger quantity compared to HIF-1α mRNA under normoxic conditions. However, they also reported that both HIF-1α and HIF-2α protein expressions were significantly and dramatically increased beginning at just 30 minutes into hypoxic exposure (1% oxygen), in a line of cells that expressed both isoforms to a high degree. These findings suggest that in particular cell lines, both isoforms are increased in response to hypoxia, but that only HIF-2α is more readily expressed under normoxic conditions.

Comparisons of the levels of HIF-1α and HIF-2α have also been made in both neural cell lines and in the brain using *in vivo* models of hypoxia. Li et al. (2009)

examined the expression of HIF-1α and HIF-2α in a line of brain cancer stem cells, glioma stem cells (GSCs), in response to normoxia and hypoxia. Interestingly, they found that only HIF-2α mRNA was induced under hypoxic conditions in GSCs. When quantifying protein, however, they found that HIF-2α protein was upregulated more readily in GSCs under both normoxic and moderately hypoxic conditions (0.2% - 5% oxygen concentrations), whereas HIF-1α protein was significantly upregulated under only the most severely hypoxic conditions (less than 1% oxygen). These results are consistent with results in peripheral cells lines that have found greater normoxic expression of HIF-2α compared to HIF-1α (O'Rourke et al., 1999; Weisener et al., 1998). Furthermore, normal (non-diseased) neural progenitor cells were found to express much greater quantities of HIF-1α mRNA compared to mRNA for HIF-2α; immunofluorescence of tissue taken from brain tumors for biopsy also showed significantly greater staining for HIF-1α compared to HIF-2α (Li et al., 2009). In contrast, some studies evaluating the expression of HIF-1α versus HIF-2α using *in vivo* models have found similar expression patters of the two isoforms in the brain in response to hypoxia. For example, Ndubuizu, Chavez, and LaManna (2009) housed rats in hypoxic conditions for three days and found similar increases in brain expression of both HIF-1α and HIF-2α protein.

Further research findings have suggested that differential upregulation of HIF-1α and HIF-2α may also be a function of the nature of the hypoxic conditions to which animals are exposed. Rahman and Thomas (2007) subjected Atlantic croaker fish to short- or long-term hypoxia, and also varied the oxygen concentrations of the tank water. They found that short-term exposure to hypoxia (3 days to 1 week) stimulated the

upregulation of HIF-1α mRNA, but not HIF-2α mRNA, in ovarian tissue. Initially, mRNA for both alpha isoforms was expressed in similar quantities, but continued hypoxic exposure of up to three weeks resulted in continual increase of HIF-2α mRNA expression. Therefore, Rahman and Thomas conclude that it is likely that HIF-1α is responsible for the initial response or adaptation to hypoxic conditions, and HIF-2α plays an increasingly important role in oxygen homeostasis with prolonged periods of hypoxic exposure. Furthermore, differential expression of HIF-1α and HIF-2α appears to be affected at least in part by the severity of the hypoxia.

Based upon the results of Rahman and Thomas (2007) and Sowter and colleagues, and according to literature regarding hypoxia (Bergeron, Yu, Solway, Semenza, & Sharp, 1999; Jewell et al., 2001; Jiang, Semenza, Bauer, & Marti, 1996; Kasiganesan, Sridharan, & Wright, 2007; Stroka et al., 2001; Taie et al., 2009; Zhang et al., 2009) and ischemia (Bergeron et al., 1999; Li et al., 2007; Li, Zhou, Calvert, Colohan, & Zhang, 2005; Marti et al., 2000; Mu et al., 2003), HIF-1α is the hypoxia-inducible factor that is most widely recognized as being intimately involved in the biological response and adaptation to low oxygen levels, and has consistently been shown to be upregulated in the periphery (Amaral et al., 2008; Ameln et al., 2005; Lloyd et al., 2003; Lundby, Gassmann, & Pilegaard, 2006; Richardson et al., 1999), and more recently in the brain following exercise (Kinni et al., 2011). Therefore, HIF-1α is the molecule of interest in the present research.

HIF-1α and VEGF

Vascular endothelial growth factor (VEGF) is a target gene for HIF-1α (Milosevic et al., 2007; Sharp & Bernaudin, 2004) and has been shown to be upregulated in response

to oxygen deprivation *in vitro* and *in vivo* (Bernaudin et al., 2002; Chavez et al., 2000; Marti et al., 2000; Zhang et al., 2009). Milosevic and colleagues (2007) created a line of HIF-1α knockout mice and found that the expression of VEGF in midbrain neural precursor cells of embryonic mice was significantly decreased compared to wild-type cells. Iyer et al. (1998) exposed embryonic stem cells to either 20% or 1% oxygen and found that VEGF mRNA expression in cells exposed to 1% oxygen was significantly decreased compared to the levels in the normoxic cells. Although expression of VEGF was not completely lost in these models, the lack of HIF-1α significantly impaired the VEGF response and suggests that HIF-1α is a strong mediator of VEGF activity.

Several studies have demonstrated increases in HIF-1α with concomitant increases in VEGF following hypoxia and/or ischemia (Chavez et al., 2000; Zhang et al., 2009). Zhang and colleagues (2009) found that HIF-1α and VEGF increased along the same timeline in cultured embryonic cortical neurons. Specifically, following exposure to oxygen and glucose deprivation (OGD), expression of both HIF-1α and VEGF mRNA were significantly increased compared to expression in normoxic cells at four hours, and levels of mRNA for both molecules remained elevated at 24 hours after OGD. Using and *in vivo* model, Chavez et al. (2000) placed rats in an environment containing 10% oxygen for various time periods and found significant increases in HIF-1α protein beginning at six hours of hypoxic exposure, and increased levels of protein persisted throughout the remainder of the study (21 days). VEGF mRNA was significantly increased beginning at 12 hours of hypoxic exposure and mRNA levels continued to rise until day four; VEGF mRNA expression did not persist, however, and were near baseline levels at day 21. VEGF protein was also upregulated,, but not until 24 hours of hypoxic exposure, and like

mRNA levels, VEGF protein returned to baseline by the 21 day time point. These findings illustrate the timeline of the expression of VEGF relative to HIF-1α, with HIF-1α protein being expressed before VEGF mRNA and finally VEGF protein (Chavez et al., 2000). This order of transcription factor and target gene upregulation has been replicated in studies of ischemia as demonstrated by Mu and colleagues (2003) who induced ischemia in rat pups and found increased HIF-1α protein expression immediately after stroke that peaked eight hours after reoxygenation and returned to control levels by 24 hours. Furthermore, VEGF mRNA was significantly upregulated beginning at two hours of reoxygenation after which mRNA levels continued to decline to baseline levels at 24 hours; VEGF protein was upregulated at four hours and also peaked at eight hours but remained elevated at the 24 hour time point (Mu et al., 2003). This timeline of events, beginning with the activation of HIF-1α followed by upregulation of VEGF mRNA and protein, from immediately after hypoxia or ischemia and persisting until for several hours or days, is in agreement with studies showing significant angiogenesis occurring a few days following hypoxic or ischemic insult (Boreo et al., 1999; Harik et al., 1996; LaManna et al., 2004; Marti et al., 2000).

Finally, exercise studies have also shown significant changes in HIF-1α expression along with changes in VEGF mRNA expression in the muscles of exercising organisms. Ameln et al. (2005) observed significant increases in HIF-1α protein immediately after knee extension exercise in the muscles of human subjects, and VEGF mRNA was also increased significantly beginning 30 minutes following exercise and reaching maximal levels at the two hour time point. In additions, whereas HIF-1α levels remained significantly elevated through the final measurement at six hours post-exercise,

VEGF mRNA decreased from two to six hours after exercise to levels not significantly different than resting baseline measures (Ameln et al., 2005). These findings are in agreement with the timeline described above with HIF-1α expression increasing first, followed by the expression of VEGF mRNA at a later time.

HIF-1α in pathological and normal physiological processes.

HIF-1α and hypoxia

Exposing cell lines, tissues, or whole animals to hypoxia is a method with which to study the physiological response to hypoxia in both the periphery and in the brain. An environment containing about 21% oxygen is considered an environment with normal oxygen tension (Lindeburg, 2006). Therefore, many research paradigms have utilized apparatuses that significantly decrease environmental oxygen levels. In this way, researchers have been able to vary both the degree of hypoxia and the amount of time the cell, tissue, or animal is exposed to the hypoxic environment, thus yielding an abundance of data regarding the characteristics of the hypoxic response.

A chamber with a drastically reduced oxygen concentration of 0.01% was used to induce hypoxia in osteoblastic cells (Kim et al., 2002). After four to six hours of exposure to the hypoxic environment, Western blotting showed a significant increase in HIF-1α protein, and also a significant upregulation of two VEGF isoforms. Similarly, Jiang, Zheng, Leung, Roe, and Semenza (1997) showed that upon exposure of human hepatoma cells to 1% oxygen for 24 hours, there was a significant increase in both HIF-1α protein and HIF-1β protein. Wang, Jiang, Rue, and Semenza (1995) also successfully induced increased expression of both HIF-1α and HIF-1β RNAs and proteins in human hepatoma cells that were placed in an environment of 1% oxygen for extended periods of

time. In another study, researchers exposed human umbilical vein endothelial cells (HUVECS) to hypoxia (1% oxygen) for varying amounts of time and found that HIF-1α protein was significantly increased (Kawanami et al., 2009). In another study with varying levels of hypoxia, Jiang and colleagues (1996) found that exposure to varying degrees of hypoxia, between 6% and 0.5% oxygen, HIF-1α protein levels increased significantly in human cervical cancer cells. Another study using five different cell lines found significantly increased DNA binding of activity of HIF-1 and significantly increased levels of HIF-1α protein (Wenger, Kvietikova, Rolfs, Gassmann, and Marti, 1997). These *in vitro* studies, utilizing various peripheral cell lines, demonstrate that exposure to severely oxygen-deprived environments is enough to induce significant changes in cellular physiology; similar studies using cortical cultures have also shown that HIF-1α mRNA and protein are significantly upregulated following exposure to hypoxia, therefore suggesting that cells in the central nervous system respond similarly to decreases in oxygen availability. Methodologically, it is important to note the severity of the oxygen deprivation in these studies, as studies using animal models of hypoxia tend to utilize much less severe hypoxic conditions (Bergeron et al., 1999; Chavez et al., 2000; Wiener, Booth, & Semenza, 1996), but are nonetheless able to arrive at the same conclusions.

Similar to studies using cell lines to study the physiological activity of HIF-1α, studies using live animals utilize chambers in which the oxygen levels can be manipulated; however, in order to ensure the survival of the animal for varying times following hypoxic exposure, conditions typically range from 5% oxygen to 10% oxygen concentrations (Bergeron et al., 1999; Chavez, et al., 2000; Wiener et al.,1996). Rats

exposed to an environment of 7% oxygen for one hour had significantly elevated levels of HIF-1α mRNA in several tissues including brain (Wiener et al., 1996). Bergeron et al., (1999) also exposed animals to hypoxia (6% oxygen) for four and a half hours and found significant increases in both HIF-1α and HIF-1β protein expression in the cingulate cortex. Similarly, upon exposure to 10% oxygen for time periods ranging from six hours to three weeks, Chavez et al. (2000) found significant increases in HIF-1α protein in animals exposed to hypoxia for six hours up to 14 days with HIF-1α levels returning to baseline in animals exposed for 21 days. In summary, these results suggest that in response to hypoxia, rapid increases in HIF-1α protein occur and are maintained for several days, and as suggested by the finding of Chavez et al. (2000), the levels return to baseline after a significant amount of time has elapsed. This return to baseline levels of HIF-1α protein may be the result of long-term adaptation and possibly a negative feedback mechanism whereby a "stopping" mechanism is triggered so as not to maintain unnecessary expression of hypoxia-inducible genes and molecules.

HIF-1α and ischemia.

In cases of ischemic stroke, blood flow to the affected region of the brain is blocked or significantly decreased which results in hypoxia (Lloyd-Jones et al., 2009). Under these conditions, homeostatic mechanisms are initiated in an attempt to maintain or restore normal oxygen tension. With the ability to affect downstream changes in neuroprotective molecules such as VEGF and EPO, the activity of HIF-1α is of great significance in ultimately maintaining the integrity of the affected brain region, not to mention the functionality of the injured cells (Semenza, 2000a). Several lines of research regarding the activity of HIF-1α following ischemia have shown significant upregulation

of the transcription factor in a variety of stroke models (Bergeron et al., 1999; Marti et al., 2000; Mu et al., 2003).

In an adult rat model of focal cerebral ischemia, both HIF-1α mRNA and protein were upregulated relatively quickly following induction of ischemia (Bergeron et al., 1999). Specifically, HIF-1α mRNA was found to be significantly upregulated in the penumbra just four hours following treatment, and remained elevated until 20 hours post-ischemia. Additionally, Bergeron and colleagues observed significant increases of mRNA in the cingulate and retrosplenial cortices up to 20 hours following treatment. In addition to their ischemia studies, the authors also quantified basal levels of HIF-1α and HIF-1β mRNAs in the hippocampus, and found a high level of expression for both isoforms. These findings indicate that the hippocampus and the surrounding areas of the brain (entorhinal cortex, cingulate cortex, etc.) readily express HIF-1α and HIF-1β under conditions of normal oxygen tension and in ischemia. Therefore, in terms of expression of HIF-1α, the hippocampus is a reasonable target structure for the current study and HIF-1α should be present in all animals regardless of the group to which the animals are assigned.

Unilateral occlusion of the middle cerebral artery (MCA) in neonatal rats was also successful in upregulating HIF-1α protein (Mu et al., 2003). Although Bergeron et al. (1999) found significant upregulation of HIF-1α mRNA at four hours post-ischemia that declined by 20 hours in several brain regions including the hippocampus, the timeline of HIF-1α protein expression is similar to the findings of Mu and colleagues (2003) who measured significant increases of HIF-1α protein just four hours following ischemia which declined at 24 hours following infarct. Although neonatal animals may not be

physiologically identical to adult animals, these data show that HIF-1α mRNA and/or protein are reliably increased in the brain following induction of ischemia.

Short-term bilateral occlusion of the carotid arteries has also been shown to dramatically increase the expression of HIF-1α (Li et al., 2005). Results from immunohistochemistry and Western blotting revealed significant increases in HIF-1α in the hippocampus of rats subjected to 10 minutes of global ischemia. The increases in HIF-1α protein were observed six hours after ischemia and peaked between 48 and 96 hours, and started to decline by seven days post-ischemia. Unlike the findings of Bergeron et al. (1999), and Mu et al. (2003), Li and colleagues have demonstrated a slower rise and fall of HIF-1α levels. It is possible that the bilateral carotid artery paradigm, being a much more global ischemic event, could result in a greater degree of apoptosis when compared to the unilateral MCA occlusion. As a result, significant increases of HIF-1α would be delayed due to the decrease in viable neurons, and due to the pervasiveness of the injury, HIF-1α would be expected to remain elevated for a larger portion of time as the brain attempts to revert to a state of homeostasis. Another study did find that HIF-1α protein levels remained elevated up to seven days following an ischemic event (Chavez & LaManna, 2002). However, in contrast to the findings of Li and colleagues, this study found that HIF-1α was significantly upregulated just one hour after ischemia. The timeline for the expression of HIF-1α protein and mRNA levels relative to stroke should continue to be investigated based on the differences in findings among studies.

HIF-1α and exercise.

By its very nature, exercise puts strain on tissue oxygen homeostasis and has therefore also been studied with regard to its effects on the expression of hypoxia-inducible factors in both human and animal subjects (Ameln et al., 20005; During & Cao, 2006; Fabel et al., 2003; Lundby et al., 2006; Kinni et al., 2011). In the periphery, HIF-1 α has been found to be activated soon after an acute exercise experience. Ameln et al. (2005) found significantly increased levels of HIF-1 α protein in the vastus lateralis muscles of human subjects that had completed 45 minutes of knee-extension exercises. This increase in protein expression was detected in muscle biopsies taken immediately after exercise completion, and protein levels remained significantly increased compared to control (pre-exercise) biopsies up to 360 minutes post-exercise. However, these authors noted that there was no significant change in HIF-1α mRNA in response to exercise. In partial support for the findings of Ameln and colleagues, Lundby et al. (2006) also failed to find a significant increase in HIF-1α mRNA at early time points after exercise - immediately after exercise or two hours post-exercise - in the vastus lateralis muscles of human subjects who had completed a three-hour knee extension exercise session . However, they did report a significant increase in mRNA expression at the six hour post-exercise time point. These findings indicate that HIF-1α mRNA and HIF-1α protein expression may have different temporal profiles following exercise. Whereas HIF-1α protein was found to be significantly upregulated quickly after exercise, the increase in mRNA expression was delayed until several hours following exercise cessation. Logically, HIF-1α mRNA would be expected to increase before significant changes in protein expression, but the findings of these two studies refute this order of

events. As Lundby and colleagues did not measure protein expression, it is impossible to compare the timelines of mRNA versus protein expression. Furthermore, the absence of a significant difference in mRNA expression in Ameln et al.'s (2005) study may highlight the vast differences in exercise protocol: Lundby et al. employed a three hour bout of exercise while the participants in the study by Ameln and colleagues exercised for only 45 minutes. The significant difference in exercise duration could possibly explain the different mRNA findings. It is also possible that HIF-1α mRNA and protein are expressed to different degrees in response to exercise, and that expression of either message or protein is not required for the induction of downstream events by HIF-1α (Wenger et al., 1997).

A few studies have examined the effects of exercise on the expression of HIF-1α protein and mRNA in the brains of animals (During & Cao, 2006; Fabel et al., 2003; Kinni et al., 2011; Lopez-Lopez et al., 2004). Fabel and colleagues (2003) failed to find a significant increase in total RNA for HIF-1α in the hippocampus of animals that had voluntarily exercised on running wheels for three days. In contrast, and in utilizing an arguably less intense exercise paradigm, During and Cao (2006) did find significantly higher levels of HIF-1α RNA in the hippocampus of animals trained in the MWM compared to animals housed in enriched environments or in standard housing. This finding is interesting in that the total aerobic activity of animals trained in the MWM would be expected to be less than voluntarily exercising animals (as utilized by Fabel and colleagues, 2003) or animals living in an enriched environment. As such, it is surprising that MWM-trained animals would display higher HIF-1α RNA than their enriched housing cohorts, or than voluntarily exercising animals.

Experimenter control of aerobic activity is greater when utilizing forced exercise paradigms compared to voluntary exercise or enriched housing conditions, and therefore seems particularly appropriate for the investigation of whether exercise alone is sufficient to cause an increase in HIF-1α. Lopez-Lopez and colleagues (2004) exposed mice to either three or 30 days of treadmill exercise and did find a significant increase in levels of HIF-1α protein in the brain. Similarly, a very recent study investigated the differences in expression of HIF-1α and various other metabolic proteins and mRNAs in the brains of rats in response to different exercise paradigms, specifically forced treadmill exercise versus voluntary wheel running (Kinni et al., 2011). Animals were allowed free access to a running wheel for three weeks while another group of rats was exposed to treadmill exercise for the same three week period. The authors reported significant increases in both HIF-1α protein and mRNA in both exercising groups compared to sedentary control animals. Furthermore, the effect was greater in animals exposed to forced exercise such that HIF-1α mRNA and protein levels were significantly greater in animals forced to run than levels in voluntarily running animals. This pattern was also observed in analysis of other metabolic mediators including GLUT-1, GLUT-3, lactate dehydrogenase (LDH), and phosphofructokinase; both protein and mRNA of all of these molecules were significantly greater in exercising animals than in control animals, and forced exercise animals had expression levels that were significantly greater than voluntarily exercising animals. These findings suggest that aerobic activity can induce in the expression of HIF-1α. Furthermore, differences in the type of exercise, voluntary and presumably intermittent exercise versus sustained aerobic exertion, result in different magnitudes of HIF-1α protein and mRNA expression.

The findings of Kinni et al. (2011) are, however, surprising when considered with the findings of studies investigating the expression of HIF-1α target molecules and their receptors. As discussed above, mRNA and protein expression of the HIF-1α target, VEGF, in the skeletal muscles of animals exposed to exercise seems to increase rapidly and then return to baseline levels even if the animals continues to exercise for a prolonged period of several weeks (Amaral et al., 2008; Lloyd et al., 2003; Richardson et al., 1999). For example, Amaral et al. (2008) found significant increases in VEGF protein in normal exercising animals after three days of treadmill running, but not after 13 weeks of running. In addition, Richardson et al. (2000) found that human participants that had undergone eight weeks of training on a knee-extension exercise had a significantly smaller expression of VEGF mRNA after a single bout of exercise compared to participants that had not undergone the eight weeks of pretraining, suggesting that long term exercise does not continue to support increased VEGF expression. These findings all suggest that increases in VEGF and their receptors increase shortly after the commencement of an exercise program, but that these changes in expression are transient. Given that VEGF is a target for HIF-1α and exerts actions downstream of HIF-1α accumulation and activation, it would follow that increases in HIF-1α would occur before changes in VEGF and VEGF receptor expression. Based on the findings regarding VEGF protein and mRNA expression returning to baseline levels even while maintaining physical activity, it would be reasonable to hypothesize that levels of HIF-1α would display a similar timeline of increases and returns to baseline levels, albeit presumably sooner than those observed with VEGF. As such, HIF-1α would increase rapidly after the start of exercise, but this increase would not be

maintained for prolonged training periods even with continued activity. This assumption, however, is not supported by the findings of Kinni et al. (2011) in that HIF-1α levels were found to be elevated in the brain after a prolonged period of sustained exercise training. It is likely that this discrepancy in results could be due to the fact that the periphery and the brain simply respond differently to exercise or that the dynamic between HIF-1α and VEGF expression is different in the CNS than in the periphery.

Although VEGF data from the periphery clearly shows a rapid increase in VEGF expression followed by a rapid return to control levels with continued exercise, it should be noted that the picture in the brain is not quite as clear. In the brain, some results suggest a similar VEGF expression timeline as those in the periphery. Tang et al. (2010) observed rapid increases in VEGF mRNA and protein in the hippocampus of animals just one hour after they had completed a single, one-hour treadmill exercise session. In another study, immunolabeling for the VEGF receptor Flk-1 was also shown to be significantly increased in the cerebellum and motor cortex after just two days or ten days of voluntary exercise, respectively (Bulinski et al., 2000; Thompson et al., 2000). Furthermore, these differences in receptor labeling were not found in animals that were allowed to exercise for 30 days (Bulinski et al., 2000; Thompson et al., 2000). However, and in contrast to these findings but in support of the findings of Kinni et al. (2011), Ding et al. (2006a) found significantly greater VEGF mRNA in the brains of animals that had exercised for three weeks compared to sedentary animals. Because Ding and colleagues did not evaluate the expression of VEGF early during the exercise protocol it is possible that VEGF did increase rapidly after the start of exercise, and that these changes persisted throughout the duration of the exercise. If this is the case, then Kinni et al.'s findings of

significantly elevated HIF-1α expression would make sense and possibly explain the persistence of the VEGF signal well into the exercise regimen. Future research should continue to study the HIF-1α-VEGF dynamic in the brain, and the current study hopes to add to the commentary on this subject with an analysis of the timeline of HIF-1α expression after an acute bout of exercise.

Hypoxic or ischemic preconditioning

As reviewed above, HIF-1α activity is induced in response to hypoxia and ischemia. Additionally, research has indicated that pre-exposure to sub lethal levels of hypoxia or ischemia before exposure to more severe hypoxia or ischemia is neuroprotective as indicated by decreases in cell death and mortality and increases in HIF-1α and downstream neuroprotective molecules such as VEGF, EPO, BDNF, and angiopoietin (Semenza, 2000a). This hypoxic preconditioning may be affected by a "priming" of HIF-1α during the less severe hypoxic event, resulting in a more efficient HIF-1α response during the more severe hypoxic insult (Lu, Ding, & Shi, 1999; Shao, Gao, & Lu, 2005; Taie et al., 2009).

Several studies have found evidence of neuroprotection following exposure to less severe hypoxia or stroke followed by a more severe event (Lu et al., 1999; Shao et al., 2005). Specifically, Lu et al. (1999) found that upon consecutively repeated "runs" of hypoxic exposure, the tolerance of adult mice to subsequent exposure was significantly increased compared to control mice that had not been previously placed in the hypoxia chamber. Similarly, Shao et al. (2005) found that with repeated exposure to airtight containers, up to four successive exposures total, there was a significant increase in "tolerance time" (p. 256) – defined as the time to the first gasping breath – such that

animals tolerated each successive exposure for longer than the previous trial. In addition, Shao and colleagues measured the activation of HIF-1α mRNA and protein. Compared to animals exposed to only one trial of hypoxia, those with four successive exposures had significantly greater quantities of HIF-1α mRNA in the hippocampus, but by the fourth exposure, HIF-1α mRNA levels returned to baseline and were not significantly different than control animals that were not exposed to hypoxia. However, HIF-1α protein levels were found to be significantly different in repeated exposure animals compared to both control and single-exposure animals. This suggests that with more exposure to hypoxic conditions, HIF-1α protein continues to increase and lends credence to the idea that previous hypoxic exposure increases the activity of HIF-1α, making it more readily available for activation in later instances of hypoxia or ischemia.

Using a neonatal ischemia model, Gidday et al. (1994) exposed 6-day-old rat pups to either one and a half or three hours of hypoxia, followed 24 hours later by unilateral carotid artery occlusion. In examining the hippocampus, neocortex, and striatum of animals exposed to three hours of hypoxia before the ischemic insult, the authors failed to find any evidence of injury; contrastingly, animals that did not receive preconditioning had severe injuries to the striatum and all areas of the hippocampus. In a similar study, Miller and colleagues (2001) exposed adult mice to hypoxia (11% O2) 48 hours before transient MCAO and found that pre-exposed animals had significantly smaller infarct volumes compared to non-preconditioned animals. The authors of these papers suggest that the mechanisms behind the protective effects of hypoxic preconditioning may involve altered neurotransmitter release, free radical scavenging activities, or the activation of genes and proteins (such as heat shock protein) (Gidday et al., 1994; Miller

et al., 2001). It is likely that more than one molecular mechanism or cascade is involved in hypoxia-induced neuroprotection, but it is possible that HIF-1α is involved as this protein is known to regulate the genes of neuroprotective and angiogenic molecules (Semenza, 2000b).

Bernaudin and colleagues (2002) were interested in the aspect of neuroprotection involving the activation of target genes of HIF-1α following pre-stroke exposure to hypoxia. They found that six hours of exposure to hypoxia (8% O_2) 24 hours before focal permanent ischemia resulted in significantly decreased brain infarct volumes compared to adult mice that had not been previously exposed to hypoxia. In addition, HIF-1α protein expression in the brain, and the expression of EPO and VEGF mRNA and protein was also significantly upregulated. These results provide evidence of neuroprotection by way of HIF-1α, EPO, and VEGF expression, and are similar to results of a study utilizing a neonatal rat ischemia model that found that three hours of exposure to hypoxia (also 8% O_2) before permanent carotid artery occlusion resulted in significantly increased protein expression of several HIF-1α target genes including glucose transporter-1 (GLUT-1), phosphofructokinase (PFK), aldolase (ALD), and lactate dehydrogenase (LDH)] (Jones & Bernaudin, 2001). Although Jones and Bernaudin did not measure EPO and VEGF, their findings of increases in several molecules required for energy metabolism provide further evidence that neuroprotection conferred by exposure to hypoxia before a more severe hypoxic or ischemic event may be due to increases in HIF-1α and the downstream regulation of target genes.

As described above, hypoxic preconditioning has been found to provide protection from a subsequent, more severe hypoxic insult or subsequent ischemia.

Similarly, studies have also found that pre-exposure to mild ischemia, rather than hypoxia, followed by a more severe ischemic event is neuroprotective (Kirino, Tsujita, & Tamura, 1991). Gerbils that had undergone a short period of ischemia one day prior to being subjected to a longer ischemic event were found to have significantly less damage to the CA1 area of the hippocampus compared to animals that had undergone only the longer event. Kirino and colleagues also noted that with increased time between the short exposure and the long exposure to ischemia, the degree of damage in the hippocampus was decreased with longer delays compared to shorter delays between the less severe and more severe events.

The phenomenon of hypoxic and ischemic preconditioning and its ability to confer neuroprotection following a more severe event suggests that the brain is adapting to the changes induced by hypoxia and/or ischemia. Hypoxia research has shown that upon acute exposure to hypoxia, cerebral blood flow and glucose metabolism increases (Beck &Krieglstein, 1987; LaManna, Chavez, & Pichiule, 2004). Furthermore, after prolonged exposure to hypoxia the partial pressures of oxygen in veins and arteries return to normoxic levels (Chavez, Agani, Pichuile, & LaManna, 2000; Dunn, et al., 2000; LaManna et al., 2004; LaManna, Vendel, & Farrell, 1992), the transport of glucose across the blood brain barrier increases (Harik et al., 1996; LaManna et al., 2004), and most importantly in the context of the present research, the vasculature of the brain begins to remodel with the addition of new capillaries as well as a decrease in the distance between capillaries (Lauro & LaManna, 1997; LaManna et al., 2004).

The adaptive morphological changes that occur in the brain under conditions of mild acute or chronic hypoxia, therefore, mimic some of the morphological changes that

occur in the brain in response to exercise, such as increased blood flow (Swain et al., 2003) and angiogenesis (Black et al., 1990; Sikorski et al., 2008; Ekstrand et al., 2008; van der Borght et al., 2009). Therefore, it is reasonable to hypothesize that the adaptive mechanisms utilized by the brain to maintain oxygen homeostasis during exercise or in conditions of hypoxia operate by way of similar pathways. Furthermore, the similarities between the effects of hypoxia and exercise upon brain morphology and chemistry, and the phenomenon of neuroprotection via hypoxic preconditioning, can explain the findings that exercise too can be neuroprotective in cases of exposure to hypoxia or ischemia (Ding et al., 2004a).

Pharmaceutical manipulation of HIF-1α.

As described above, VEGF is involved in exercise induced angiogenesis and is an HIF-1α target. As such, inhibition of HIF-1α would be expected to cause a significant decrease in the expression of VEGF and therefore would inhibit angiogenesis. Indeed, some research has demonstrated that inhibition of HIF-1α does have a deleterious effect on the expression of VEGF in both *in vitro* and *in vivo* assays (Svensson, Azarbayjani, Backman, Matsumoto, & Christofferson, 2005; Yoshida et al., 2010; Zhang et al., 2008). SomeHIF-1α inhibitors belong to a class of drugs called cardiac glycosides, which includes digoxin, ouabain, and proscillaridin Zhang and colleagues (2008) exposed different cancer cell lines to varying oxygen levels (1% or 20% oxygen) in the presence of digoxin, ouabain, and proscillaridin and found that all three drugs successfully blocked the expression of HIF-1α protein in cells exposed to hypoxia. Additionally, when they injected tumor generating lymphocyte cells into immunodeficient mice and treated the animals with digoxin, tumor growth was significantly attenuated. As VEGF plays a role

in tumor growth, Zhang and colleagues (2008) also assessed the expression of VEGF in response to treatment with digoxin and found that VEGF mRNA expression was significantly decreased in the tumors. In summary, these findings demonstrate that digoxin inhibits the HIF-1α response to hypoxia *in vitro* and in tumor growth *in vivo*, and this decrease in HIF-1α protein expression is accompanied by decreased expression of VEGF.

Other studies have investigated the effects of digoxin in central nervous system tissue (Svensson et al., 2005; Yoshida et al., 2010). For example, Yoshida and colleagues evaluated the effects of digoxin on "choroidal neovascularization" following induction of ischemia in the retina of mice (p. 1760). Results showed that in animals treated with PBS (control), retinal ischemia induced a significant increase in HIF-1α protein expression, but in animals that underwent ischemia and were pre-treated with digoxin, expression of HIF-1α protein was significantly decreased. Furthermore, they showed that mRNA expression of several HIF-1α targets, such as VEGF, GLUT-1, and EPO, were decreased in mice treated with digoxin before retinal ischemia. Svensson and colleagues (2005) demonstrated that the growth of some xenografted neural cancer cells was significantly inhibited upon treatment with digoxin. However, these authors also note that it is possible that the inhibition of tumor growth may not be a result of decreased angiogenesis, as indicated by findings of a follow-up study that tissue taken from tumor cells treated with digoxin did not have significantly different vascularity compared to control tissue. While the study by Svensson and colleagues (2005) does demonstrate that digoxin decreases neuroblastoma tumor growth, it is unclear how this growth is being inhibited. Based on the finding that angiogenesis did not change significantly in

xenografted tumors that received digoxin compared to controls, it is possible that digoxin is inhibiting tumor growth by means other than inhibition of angiogenesis. The inhibition of HIF-1α has not been studied extensively in *in vivo* models, and most research with these drugs has focused on tumor growth and survival (Svensson et al., 2005; Zhang et al., 2008). However, HIF-1α agonists have received much attention, specifically in investigations of neuroprotection.

Most clinical applications of HIF-1α involve inducing the upregulation of the transcription factor because of its downstream effects on potentially neuroprotective genes such as VEGF and EPO (Siddiq, Aminova, & Ratan, 2007). As described previously, under normoxic conditions HIF-1α is broken down through a pathway of proteasomal degradation (Sharp & Bernaudin, 2004). More specifically, HIF-1α is hydroxylated under normal oxygen conditions, and this hydroxylation depends on the presence of "di-oxygen (O_2), iron and 2-oxoglutarate" (Sharp & Bernaudin, 2004, p. 439; see also Chen et al., 2009; Harten et al., 2010). Once hydroxylated, HIF-1α binds to the von Hippl-Lindau (VHL) complex which then leads to the degradation of the subunit. Some common therapeutic targets used to increase the expression of HIF-1α and inhibit its degradation are prolyl-4-hydroxylase (PHD) inhibitors, which function to inhibit the hydroxylation the oxygen-dependent areas of the HIF-1α gene (Sharp & Bernaudin, 2004; Siddiq et al., 2007). Other agents that interfere with the binding of the VHL complex to HIF-1α include iron chelators and some heavy metal compounds (Sharp and Bernaudin, 2004). It should also be noted that these drugs can be also used in normoxic conditions to artificially induce the expression of HIF-1α and its downstream targets such

as EPO and VEGF (Chu, Beart, & Jones, 2010; Jones & Bergeron, 2001; Schneider et al., 2009).

Several studies have used PHD inhibitors, iron chelators, or heavy metal compounds to increase the expression of HIF-1α before induction of stroke or hypoxia to determine if pre-elevated levels of HIF-1α would result in neuroprotection (Chu et al., 2010; Hamrick et al., 2005; Jones & Bergeron, 2001; Schneider et al., 2009). For example, Chu and colleagues (2010) treated astrocytes extracted from mouse forebrain with the PHD inhibitor ethyl 3,4-dihydroxybenzoate (EDHB) prior to administration of oxidative injury induced by hydrogen peroxide (H_2O_2) *in vitro*. They found that treatment for either four or 24 hours prior to H_2O_2 injury conferred a significant protective effect in that a greater number of cells in culture were considered viable after injury. These authors also showed significant increases in HIF-1α protein at four hours post-injury (Chu et al., 2010). In an *in vivo* model Jones and Bergeron (2001) exposed rat pups to hypoxia or injected them with the compound $CoCl_2$ (cobalt chloride; a heavy metal compound that interferes with the ability of PHDs to hydroxylate HIF-1α; Sharp & Bernaudin, 2004) 24 hours before induction of hypoxic-ischemic injury. They found that pretreatment with either $CoCl_2$ or hypoxia resulted in significantly less brain damage compared to animals not pretreated with hypoxia or $CoCl_2$. These findings indicate that treatment with drugs known to manipulate the expression of HIF-1α has a neuroprotective effect.

Desferroxamine (DFO) is an iron chelator and is another agent used to inhibit the hydroxylation of HIF-1α by interfering with the ferrous ion found on PHDs, and which ultimately results in the inability of the PHD to hydroxylate HIF-1α (Sharp & Bernaudin,

2004). Hamrick and colleagues (2005) pretreated hippocampal neurons with DFO prior to oxygen and glucose deprivation *in vitro* and found that pretreatment led to significantly reduced cell death compared to no treatment. Furthermore, treatment with DFO plus an HIF-1α antisense abolished the protective effect of DFO pretreatment, suggesting that DFO acts by way of HIF-1α to induce neuroprotection in OGD preparations (Hamrick et al., 2005). Chu et al. (2010) reached similar conclusions upon pre-treating astrocytes with DFO for four or 24 hours before H_2O_2 injury. They found that DFO significantly reduced the number of destroyed cells upon injury. These results indicate that similar to PHD inhibitors and heavy metal compounds, DFO is also an effective preparation for affecting neuroprotection following hypoxic or ischemic conditions.

HIF-1α can also be upregulated artificially by injection of PHD inhibitors, avoiding the necessity of exposing cell cultures or animals to hypoxia or ischemia. Chu and colleagues (2010) showed that treatment with EDHB alone with no induction of H_2O_2 injury caused significant increases in HIF-1α protein expression just two hours after treatment and this expression reached maximum levels four hours after EDHB treatment (Chu et al., 2010). These authors also treated cells with DFO alone and found significant increases in HIF-1α expression, and surprisingly, the elevated expression persisted for 24 hours following treatment. In another study, Schneider et al. (2009) injected rat pups with FG-4497, another PHD inhibitor, and extracted brain tissues six hours later. They found significant increases in HIF-1α protein as well as increased expression of VEGF and EPO mRNA. Finally, in another *in vivo* preparation, mice were injected with FG-4497 and six hours later levels of HIF-1α protein and VEGF and EPO mRNA were

significantly increased compared to animals injected with vehicle solution (Adamcio, Sperling, Hagemeyer, Walkinshaw, & Ehrenreich, 2010).

Another PHD inhibitor that has been shown to increase the expression of HIF-1α *in vivo* is dimethyloxylylglycine (DMOG). Ogle, Gu, Espinera, & Wei (2012) injected DMOG intraperitoneally and found significantly increased levels of HIF-1α protein 12 and 24 hours after injection. When DMOG was administered 30 or 60 minutes after stroke, the authors found significantly decreased the infarct volume, and significantly increased HIF-1α expression, VEGF mRNA levels, and cerebral blood flow. These findings suggest that treatment with a PHD inhibitor can increase levels of not only HIF-1α but also of VEGF. Due to its availability and the rapid upregulation of HIF-1α following injection, and findings of the angiogenic factor VEGF, DMOG was used as the HIF-1α agonist in Experiment II of this dissertation.

Taken together, the pharmaceutical manipulation of HIF-1α via substances that interfere with the hydroxylation of the subunit by PHDs confers significant neuroprotection from hypoxic and/or ischemic injuries. Furthermore, the findings that these drugs increase the expression of HIF-1α along with its downstream targets suggests that the mechanism by which cells survive injury is mediated at least partially by HIF-1α. These experimental models provide scientists the important means with which to study the ways by which manipulation of HIF-1α expression leads to neuroprotection following injury. Interestingly, other applications of HIF-1α agonists have recently been reported, namely these drugs have been evaluate in the context of cognitive enhancement.

HIF-1α and cognition

There is some evidence that expression of HIF-1α may lead to improved performance on cognitive tasks. A recent study pharmacologically manipulated the expression HIF-1α via a PHD inhibitor and tested mice on various behavioral tasks (Adamcio et al., 2010). They found that administration of the PHD inhibitor FG-4497 caused a significant increase in HIF-1α protein in the hippocampus. Additionally, the expression of mRNA for both VEGF and erythropoietin (EPO) was significantly increased. Behaviorally, they found that mice injected with FG-4497 performed significantly better than placebo-treated mice in contextual fear conditioning that took place either three or four weeks after the last injection (but not at one week post-treatment). The authors hypothesize that this improvement in cognitive performance may be mediated by the increases in EPO and or VEGF (Adamcio et al., 2010). These assumptions are supported by other research that has shown that treatment with EPO lead to improvements in memory performance (Adamcio et al., 2008). Specifically, Adamcio and colleagues (2008) injected mice with EPO for three weeks and tested them on a variety of behavioral activities. Animals treated with EPO performed significantly better than control animals in contextual fear conditioning (but not cued fear conditioning) at both one week and at three weeks post-treatment. Moreover, they also demonstrated that hippocampal LTP was significantly enhanced in animals injected with EPO suggesting that EPO promotes changes in synaptic function and ultimately facilitation of cognitive performance. El-Kordi, Radyushkin, and Ehrenreich (2009) also showed that operant and discriminant conditioning tasks were facilitated following three weeks of EPO injections in mice.

The findings of the more recent study by Adamcio and colleagues (2010) that there is a delay (three or four weeks) between treatment with the PHD inhibitor and behavioral facilitation in a learning task, makes sense in light of the results of the previous study by this group where they found that performance in a contextual fear conditioning task was improved just one week after treatment with EPO. In the 2010 study, treatment with FG-4497 increased HIF-1α expression, followed by increases in EPO and VEGF mRNA. The earlier study showed that treatment with EPO facilitated performance just one week after the last injection. Taken together these findings indicate that EPO may be the main mediator in the facilitation of learning following treatment. The time lag between the manipulation of HIF-1α, and improvements in behavioral facilitation are possibly due to the time lag between increased expression of HIF-1α, the induction of EPO expression, and eventually the growth of new capillaries which is one of the functions of EPO (Ribatti, Vacca, Roccaro, Crivellato, & Presta, 2003).

Because HIF-1α targets genes that are imperative for angiogenesis, such as VEGF and EPO (Brines & Cerami, 2005; Fong, 2009), it is plausible to assume that angiogenesis is a mediator of the cognitive enhancement observed by Adamcio's group (2008, 2010). This assumption is supported by the findings of Kerr and Swain (2011) who showed that exercise-induced angiogenesis but not neurogenesis is necessary for acquisition and facilitation of performance in the MWM. Another purpose of our study will be to compare and contrast exercise-induced facilitation of learning with any facilitation caused by pharmaceutical upregulation of HIF-1α.

HIF-1α Timeline

In general, the upregulation of HIF-1α in response to hypoxia occurs relatively quickly following hypoxic exposure. Stroka et al. (2001) found that brain levels of HIF-1α protein in mice were significantly increased after just one hour of exposure to a hypoxic environment (6% oxygen). They also showed that HIF-1α levels in the brain peaked between four and five hours in the hypoxic environment, and that HIF-1α levels returned to baseline between nine and 12 hours after exposure. In localizing the HIF-1α protein, Stroka et al. (2001) found strong staining in several areas of the brain, including the dentate gyrus of the hippocampus and the hippocampus itself. Surprisingly, a study conducted by Jewell et al. (2001) showed significant increases in HIF-1α protein in the nucleus of cells *in vitro* after just two minutes of hypoxic exposure. Furthermore, Jewell and colleagues showed that drastic increases of HIF-1α protein continue for the first 30 minutes of exposure, reach peak levels at 60 minutes, and can be maintained for up to four hours of exposure to low-oxygen conditions. To further support the finding of such rapid changes in HIF-1α concentrations, they also exposed cells to hypoxia for one hour, followed by various times of reoxygenation. Similarly to the rapid increase in HIF-1α, upon exposure to normoxic conditions, HIF-1α nucleic protein levels were quickly decreased after four minutes, and levels of HIF-1α had decreased to the point of being almost undetectable at 32 minutes after re-exposure to an environment with normal oxygen concentration.

Similarly rapid increases in HIF-1α mRNA rather than protein have also been reported (Weiner et al., 1996; Zhang et al., 2009). Zhang and colleagues (2009) found a rapid upregulation of not only HIF-1α mRNA expression, but also VEGF mRNA

expression when neurons were exposed to oxygen and glucose deprivation (OGD, 0% oxygen) *in vitro*. Specifically, they found that HIF-1α mRNA expression began to increase following two hours of exposure to OGD conditions, and that VEGF mRNA increased following four hours of OGD treatment. Both HIF-1α mRNA and VEGF mRNA had returned to baseline levels by 12 hours post-exposure. These authors also investigated protein expression and found that levels of HIF-1α protein is rapidly increased in response to hypoxic conditions, and protein expression followed the same timeline as HIF-1α mRNA expression with significant upregulation beginning at four hours, peaking at eight hours, and returning to control levels at 12 hours.

Whole animal studies of the timeline of the upregulation of HIF-1α also indicate a rapid response of the brain to hypoxia. Weiner and colleagues (1996) exposed rats and mice to one hour of hypoxia in a chamber with 7% oxygen and found significant increases in HIF-1α mRNA in whole brain compared to animals kept in normoxic conditions. Bernaudin et al. (2002) exposed animals to varying time periods in a normobaric chamber with 8% O_2. Levels of HIF-1α protein were increased at the earliest time point (1 hour) and reached maximum levels at three hours, but were still significantly increased after 6 hours of exposure. These findings also point to a rapid homeostasis-maintaining response to the decrease of oxygen availability to the brain.

Studies utilizing a paradigm of prolonged hypoxic exposure have yielded similar findings as acute hypoxic exposure paradigms. Chavez and colleagues (2000) exposed rats to hypobaric hypoxia with and oxygen concentration of 10% for varying time periods ranging from six hours to three weeks of continuous hypoxia. They found that HIF-1α protein was significantly increased following six hours of exposure, and the levels

remained increased up to 14 days of hypoxia; however, by day 21 of hypoxic exposure, HIF-1α protein levels were no longer significantly different than baseline levels. Chavez et al. (2000) suggest that this return to normal levels is due to the restoration of normal oxygen tension in the hypoxic animals, and indicates that the brain is able to adapt to prolonged changes in oxygen levels, and no longer requires the actions of HIF-1α to maintain normal physiological functioning. Furthermore, to determine if the activation and plateau of HIF-1α expression in animals that had remained in the hypoxic environment for three weeks changed the ability of the brain to adapt to subsequent hypoxic exposure, Chavez et al. (2000) exposed these animals to an additional four hours of either 10% or 8% normobaric oxygen. Because the 10% oxygen environment had been used previously, animals exposed to this environment for the additional four hours did not show an upregulation of HIF-1α, but animals exposed to the less-oxygenated environment did have significant upregulations of HIF-α. These findings suggest that although the brain had adapted to the 10% oxygen conditions and the upregulation of HIF-1α was not necessary to maintain oxygen homeostasis, the HIF-1α response was still activated when animals were challenged with a further decrease in oxygenation, and this response was evident within the four hours of exposure indicating the that the brain retained the ability to rapidly respond to the conditions despite being previously acclimated to a low-oxygen environment (Chavez et al., 2000).

Research paradigms using ischemia induced via transient or permanent, and focal or global cerebral artery occlusion has also delineated a timeline for the expression of HIF-1α. For example, Bergeron and colleagues (1999) found that rats that had undergone permanent focal ischemia had significantly increased levels of HIF-1α mRNA from 7.5

hours post-ischemia with continued increases up to 20 hours following ischemic induction. Mu and colleagues (2003) occluded the middle cerebral artery of rat pups for one and half hours and found significantly increased HIF-1α protein expression at just four hours post-MCA occlusion. HIF-1α protein levels peaked at eight hours and returned to normal levels 24 hours after ischemia. The data from these studies of HIF-1α induction by stroke also indicate that the upregulation of HIF-1α protein or mRNA occurs shortly after the insult.

Pharmaceutical agents that artificially increase HIF-1α, such as PHD inhibitors and iron chelators, also upregulate HIF-1α fairly quickly. Chu et al. (2010) showed that treatment of astrocytes in culture with EDHB significantly increased expression of HIF-1α after just two hours post-treatment and that expression peaked at four hours. Similarly, they also showed that treatment with DFO increased expression of HIF-1α protein after two hours, but that levels of HIF-1α remained elevated at both eight and 24 hours after treatment (Chu et al., 2010). Furthermore, Schneider et at. (2009) and Adamcio and colleagues found that six hours after injections of FG-4497 HIF-1α protein and VEGF and EPO mRNA were significantly increased in treated animals compared to animals injected with vehicle.

As detailed above, in addition to hypoxic exposure or ischemic insult, exercise has also been shown to upregulate HIF-1α in both the periphery and the brains of exercising humans and animals, respectively, and this upregulation also occurs relatively quickly after exercise. Ameln and colleagues (2005) found significant increases in muscular HIF-1α protein measurements immediately after human subjects completed 45 minutes of knee extension exercises. Furthermore, this increase in HIF-1α remained

elevated for six hours after exercise. Lundby et al. (2006) found an increase in HIF-1α mRNA in human subjects, but this increase was not as rapidly detected as the protein increase reported by Ameln et al. as levels of mRNA were not significantly different until six hours post-exercise. These results show that the molecular mechanisms that are activated to adapt to conditions of increased oxygen demand are begun fairly rapidly providing tissue the ability to regain and maintain normal functioning.

To our knowledge, very few studies have analyzed the activity of HIF-1α in response to exercise in the brain (During & Cao, 2006; Fabel et al., 2003; Kinni et al., 2011; Lopez-Lopez et al., 2004), and these studies have yielded highly variable results. For example, Fabel and colleagues (2003) failed to identify any change in the total RNA expression of HIF-1α in the hippocampus of animals that had voluntarily exercised for three days. However, During and Cao (2006) found significant upregulation of HIF-1α RNA in the hippocampus of animals that had been trained in the MWM. In the context of Fabel et al.'s findings, these findings are surprising in that the MWM does not require extensive physical activity, whereas voluntary exercise as used in the Fabel et al. study would presumably require more exertion. As such, expression of HIF-1α would be expected to be upregulated to a higher degree after running compared to after swimming training. These somewhat contradictory findings warrant further investigation into determining which types of exercise paradigms are appropriate for study of HIF-1α.

A very recent study has provided evidence that exercise is sufficient to cause the upregulation of HIF-1α in the brains of animals. Kinni and colleagues (2011) compared the effects of voluntary and forced exercise on the expression of several metabolic proteins including HIF-1α in the brains of adult rats. Animals either ran on a treadmill

for five days per week for three weeks or were allowed free access to a running wheel for three weeks. Kinni et al. found significant increases in both HIF-1α mRNA and protein in whole brain samples from rats that completed either type of exercise compared to sedentary control animals. However, they did note that the exercise-induced increase in HIF-1α and protein expression was significantly larger in animals that were forced to exercise compared to voluntary exercisers. These findings confirm the hypothesis of the current study that exercise is sufficient to cause an upregulation of HIF-1α in the brain of exercising animals, but do not provide a timeline for exercise-induced induction of HIF-1α expression in the brain. Furthermore, it is unclear from the findings of Kinni et al. whether or not significant increases in the transcription factor occur quickly after a single bout of exercise.

The purpose of this dissertation was, in part, to replicate and expand upon the findings of Kinni and colleagues (2011) through the use of slightly different methodology. Whereas Kinni et al. measured HIF-1α protein and mRNA levels in whole brain extracts, the current study used immunohistochemistry to quantify protein and also to find local expression of HIF-1α in the hippocampus of animals that will exercise for three weeks. Furthermore, the current study attempted to determine if a single bout of exercise is enough to induce HIF-1α expression in the hippocampus, focusing on time points early after the cessation of exercise.

Methods

Experiment I

Kerr and Swain (2011) found that one of the first responses of hippocampal neurons to an exercise regimen is apoptosis which is followed shortly thereafter by

angiogenesis and neurogenesis. It is possible that exercise is causing mild hypoxia in the brain resulting in apoptosis shortly after the commencement of an exercise regimen. Therefore, the purpose of Experiment I was to determine if a single bout of forced exercise is sufficient to induce a hypoxic environment in the brain severe enough to prompt the upregulation of HIF-1α. Additionally, Experiment I investigated the timeline of exercise-induced expression of HIF-1α. Past research has indicated that the increase in HIF-1α in response to hypoxia takes place relatively quickly, occurring within one or two hours after exposure to hypoxia, and returning to baseline levels around nine or 12 hours following hypoxic insult (Stroka et al., 2001; Jewell et al., 2001; Zhang et al., 2009). Exercise studies have also shown significant increases in HIF-1α protein expression in muscle tissue, beginning very shortly after exercise cessation and persisting until six hours post-exercise (Ameln et al., 2005). To our knowledge, an experiment to determine the timeline of the expression of HIF-1α protein in the brain shortly after a single bout of exercise has not been conducted. Therefore, animals were sacrificed at various time points - from immediately after exercise cessation to 24 hours post-exercise to determine the timeline of exercise-induced HIF-1α expression. Finally, Experiment I attempted to replicate and expand upon the findings of Kinni et al. (2011) who found significant changes in HIF-1α mRNA and protein levels in the brains of animals that had exercised on a treadmill for three weeks. While Kinni and colleagues used measured both mRNA and protein in whole brain extracts, we analyzed HIF-1α protein in the hippocampus specifically though the use of immunohistochemistry.

Animals.

All animal procedures adhered to federal laboratory guidelines and were approved by the University of Wisconsin – Milwaukee Institutional Animal Care and Use Committee (IACUC). One hundred adult male Long Evans Hooded Rats (175-199 g) were obtained from Harlan (Indianapolis, IN) and housed individually in standard laboratory shoebox cages without access to enrichment or running wheels. Animals were maintained on a 12 hour light/dark schedule and had free access to food and water throughout the experimental protocol.

Animals were divided into two groups: exercise (EX, n = 50) and inactive (IC, n = 50). Within the EX and IC groups, animals were further divided into four groups each (n = 10 animals per group) corresponding with the time after treadmill exercise or exposure at which the animals were sacrificed: immediately (EX0, IC0), two hours (EX2, IC2), four hours (EX4, IC4), six hours (EX6, IC6). There were also two additional groups of animals that exercised or were exposed to the treadmill for three weeks (EXLT and ICLT) and sacrificed immediately after the final exercise or exposure session.

Apparatus.

Animals ran (EX groups) or were exposed to (IC groups) a Weslo Cadence DL5 treadmill. A rectangular Plexiglass box with three lanes (treadmill dimensions: 11.5 inches wide x 18.5 inches long x 5 inches deep; lane dimensions: 3.5 inches wide x 18.5 inches long x 5 inches deep) was affixed to the treadmill (see Figure 1). The Plexiglass box has perforations on all sides and a perforated cover; the sides and the cover prevented animals from running into one another or from escaping during the running procedure. The treadmill apparatus also had a space through which the animals' tails and/or limbs

could fit so that no physical injury to the tail or limbs was possible should the animal stop running. A metal grid was affixed to the end of two of the three lanes and was attached to a power supply that ran repeated trains of current (~1 mA), such that animals were administered a shock if they refused to run or slid to the back of the box. Finally, black construction paper was taped to the top of the apparatus and covered half of the box, creating a dark environment in the forward half of the Plexiglass box. This was meant to provide to the animals further incentive to run as rats prefer dark areas.

Figure 1. Experiment I treadmill apparatus with shock grid

Procedure.

Pre-exposure.

All animals underwent a six day pre-exposure protocol (see Table 1). On days one and two animals were transported in their home cages to the experimental room where the treadmill was located and handled gently for three minutes each, after which they were transported back to the animal room. On the third day, animals were transported in the same manner as on days one and two and placed in the treadmill apparatus and allowed to explore for three minutes; the treadmill track was not in motion

for this exposure, and the shock grid was not activated. On days four, five, and six animals in the exercise groups were placed in the treadmill apparatus and ran at a slow speed (0.3 mph) for three minutes. During these exposures, the shock grid was activated and administered a mild shock to the animals if they failed to run. These exposures were meant to allow the animal to learn to run on the treadmill without having to exert itself to a great extent, and to learn about the shock at the back of the Plexiglass box.

Table 1.

Handling and Treadmill Pre-Exposure Procedure

Day(s)	Activity	Duration (minutes)
1-2	Transport & handling	3
3	Treadmill exposure	3
4-6	Treadmill exposure (IC animals) or slow run (0.3mph; EX animals)	3

Exercise or exposure sessions.

On the seventh day, the animals completed the actual 20-minute exercise or exposure session. During the actual exercise session, EX animals were placed in the running apparatus and the speed of the treadmill was gradually increased over the course of five minutes (minute zero to one at 0.1mph; minute one to two at 0.2 mph, minutes two to four at 0.3 mph, minute four to five at 0.4 mph) until the target speed of 0.5 to 0.6 miles per hour (~ 15 meters/minute) was attained; the animals ran at this speed for the remaining 15 minutes of the exercise session. This running speed allowed the animals to achieve a fast jog as it was important for the animals to be exercised hard enough so that the hypothesized exercise-induced hypoxia was achieved. Following the running session,

animals were returned to their home cages until sacrifice at the above mentioned time points. Animals in the EXLT condition underwent the same exercise duration and intensity described above, but exercised for five days per week for the three week period. The two days of rest per week during the long term condition did not occur on consecutive days.

Animals in the IC conditions were placed in the treadmill apparatus for the twenty-minute session but the treadmill belt remained immobile. After exposure, IC animals were then returned to their home cages and sacrificed at the same time points as their exercising cohorts. ICLT animals were exposed to the treadmill on the same days that EXLT animals ran on the treadmill, and also had two, non-consecutive days of no treadmill exposure per week for the three week duration of the experiment.

Tissue preparation.

Immunohistochemistry.

Animals were sacrificed by immersion in a chamber pre-filled with isoflurane vapor and transcardially perfused with 200 mls of 0.1 M phosphate buffer followed by 400 mls of 4% paraformaldehyde. Animals were decapitated and the brains extracted and postfixed in 4% paraformaldehyde overnight. On the following day, the tissue was placed in a 30% sucrose and phosphate buffer solution until cryoprotected, as indicated by sufficient absorption of the sucrose solution, or the brain sinking to the bottom of the specimen jar.

Tissue was cut such that the entire dorsal hippocampus was captured within the block, and was sliced coronally using a Leica CM 3050 S cryostat (Wetzlar, Germany) at a thickness of 20 μm. Each slice was placed sequentially into one of six wells of an ice

cube tray and stored at -4°C until further processing via immunohistochemistry. The slices occupying one well of the ice cube tray was used for immunohistochemistry. In this way, one slice per 120 μm of tissue was processed such that the entire dorsal hippocampus is represented.

One well of tissue from each animal was immunolabeled using an HIF-1α antibody (AbCam, Cambridge, MA). Briefly, tissue was placed in perforated wells and washed twice (5 minutes each) in phosphate buffered saline (PBS) with azide, followed by catalysis for one hour in a solution of 0.3% hydrogen peroxide in PBS with azide. The tissue was washed twice more (5 minutes each) in PBS with azide, followed by two, 10 minute washes in a 2% serum in PBS with azide solution and blocked overnight in a solution containing 10% serum and 0.5% triton X (concentration: 10%) in PBS with azide. On the second day of processing, tissue was washed twice (5 minutes each) in PBS with azide followed by two washes (10 minutes each) in the 2% serum-PBS with azide solution. Non-perforated wells were filled with a primary antibody solution (1% serum, 0.5% triton X, 1:1000 primary antibody), and control wells were treated with a serum and triton X solution that does not contain the antibody. Tissue was incubated overnight in the primary antibody solution.

On the final day of processing, tissue was transferred back into perforated wells and washed twice each in PBS with azide and the 2% serum solution (5 minutes and 10 minutes each, respectively). After the washes, tissue was placed back into the non-perforated wells and incubated for 90 minutes with a solution containing a secondary biotinylated antibody (10% serum and 1% secondary antibody in PBS with azide) (Horse anti-mouse IgG, Vector Laboratories, Burlingame, CA). Following incubation with the

secondary antibody, tissue was transferred back into perforated wells for two washes in PBS without azide (5 minutes) and two washes (10 minutes each) in a 2% serum solution in PBS without azide. Tissue was placed in wells prefilled with avidin biotin complex (ABC) and incubated for one hour. After tissue was placed back into perforated wells, it was washed twice in PBS without azide (5 minutes/wash) and tris buffer (10 minutes/wash). Tissue was then reacted with DAB (3-3' diaminobenzidene) for five to ten minutes and washed five times in PBS without azide (5 minutes each). Following the washes, tissue was placed on gelatin coated slides and allowed to dry at room temperature overnight. Slides were cleared in a series of ethanol solutions and coverslipped. Slides were analyzed and imaged, and immunolabeling for HIF-1α was quantified.

Tissue imaging and HIF-1α quantification.

The dorsal hippocampus was imaged systematically such that the entirety of CA1, CA3, and the dentate gyrus (DG) were captured using a SPOT Insight digital camera (Version 4.5.7). More specifically, using the 40x magnification objective (Olympus BX41 microscope) the most lateral point of the hippocampus and most ventral point of the DG were identified and recorded, followed by the triangulation point of these two points, where the first image was taken. The stage was moved approximately 0.6mm in the medial/lateral direction to obtain a row of non-overlapping images. When the areas of interest were no longer present, the stage will be returned to the first point (triangulation of the most dorsal and most lateral points) and moved 0.5 mm in the dorsal/ventral plane and another medial/lateral row of images obtained. This procedure

was repeated until the entire structure was represented in non-overlapping images. See Figure 2 for an illustration of the imaging grid.

Following imaging, using two rolling dice, two images per region, per slice were randomly selected for quantification. To reduce the possibility of bias during quantification, we randomly coded images using a random number generator (Urbaniak & Plous, 2011). Image J (Rasband, NIH 1997-2012) was used to quantify the area fraction of HIF-1α-positive cells in each of the three hippocampal regions of interest. More specifically, images were transformed to 8-bit image files and the region of interest (ROI) was outlined. Then, a binary thresholding procedure was performed such that labeled cells were selected within each image and the background was subtracted leaving an image with just the labeled cells outlined in black. The ROI was superimposed back onto the newly thresholded image and the area of the particles within the region of interest was measured, from which an area fraction measurement was made using the following formula:

$$\left[\frac{\text{Area of Particles}}{\text{Area of ROI}}\right] \text{x} 100$$

Statistical analysis.

A three-way analysis of variance (ANOVA), with activity (EX vs. IC), time (0 vs. 2 vs. 4 vs. 6 vs. LT), and region (CA1 vs. CA2/3 vs. DG) as factors, was conducted to compare the mean expression of HIF-1α protein. Post-hoc tests using the least significant difference (LSD) correction was used to identify any specific group differences among animals in protein levels at the varying time points after exercise.

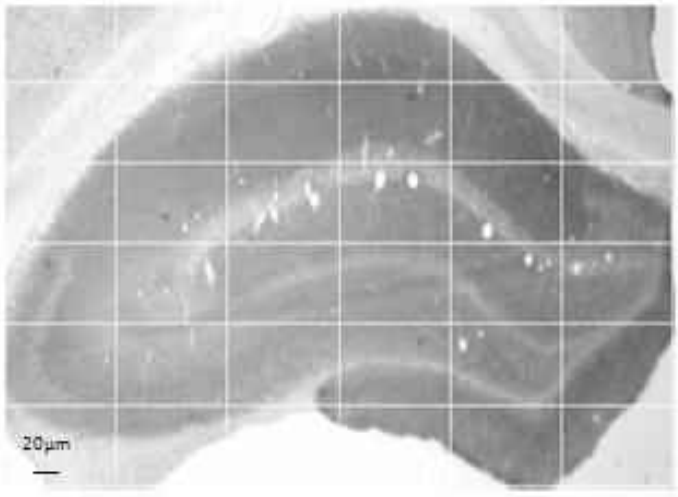


Figure 2. Illustration of a hippocampal section with the imaginary imaging grid superimposed, Magnification = 100x

Experiment II

The purpose of Experiment II was to determine the role of HIF-1α expression and/or downstream angiogenesis in behavioral performance. Because it is well-established that exercising animals perform better than sedentary animals in the learning and memory tasks (Gomez-Pinilla, et al., 2008; Griesbach et al., 2009; Kerr et al., 2010; van Praag et al., 1999; Vaynman et al., 2004), we compared the performance of exercising animals to that of animals administered the HIF-1α agonist dimethyloxylylglycine (DMOG). To supplement the behavioral findings, HIF-1α expression and blood vessel density were also quantified in order to enable comparisons between anatomy and behavior. We propose that if HIF-1α expression or downstream changes in vasculature are involved in cognitive performance, then animals administered the drug should perform similarly to exercising animals.

Animals.

All animal procedures adhered to federal laboratory guidelines and were approved by the University of Wisconsin – Milwaukee Institutional Animal Care and Use Committee (IACUC). Sixty-five adult male Long Evans Hooded Rats (175-199 g) were obtained from Harlan (Indianapolis, IN) and housed individually in standard laboratory shoebox cages without access to enrichment, with the exception of the VX animals who were allowed access to a running wheel for the seven day experimental period. Animals were maintained on a 12 hour light/dark schedule and had free access to food and water throughout the experimental protocol.

Animals were divided into five groups: a voluntary exercise group (VX, n = 13), a forced (treadmill) exercise group (FX, n = 13), a group treated with the HIF-1α agonist DMOG (DMOG, n = 13), a vehicle treated group (VEH, n = 13), and an inactive control group (IC, n = 13). Five of the animals from each group were sacrificed at the end of the experimental period, and the other eight animals underwent MWM training.

Apparatus.

Voluntary exercise.

Animals in the voluntary exercise condition were housed in cages that contained a running wheel. Half of the running wheel cages were modified shoebox cages with a hole cut out on one end through which the animal could walk to gain access to the running wheel (14 in. diameter) , which was affixed to the back of the cage (see Figure 3A). These cages were equipped with a manual counting arm that advanced the numerical display one number for each revolution the animal ran. The other half of the running wheel cages were wire-bottom cages which contained the wheel (see Figure 3B).

These wheels (13.5 in. diameter) were attached to a digital counting unit which recorded the number of revolutions each animal ran.

A. B.

Figure 3. Voluntary running wheels. A) Running wheel cage with manual counting device, B) running wheel with digital counting device.

Forced exercise.

Animals in the FX group underwent the same pre-exposure procedure described in Experiment I. The treadmill used for this study was a Pro-Form 515TX human treadmill, equipped with the same Plexiglass apparatus as described in Experiment I. Additionally, because of the abrasiveness of the treadmill track, a soft rubber Con-Tact brand liner was fitted to the treadmill track and placed directly underneath the lanes of the apparatus in which animals ran (See Figure 4). The apparatus position was adjusted to allow for animals' tails and limbs to fit between the apparatus and the customized track. This positioning ensured that animals would not be harmed while running.

Figure 4. Treadmill and treadmill apparatus for Experiment II. A soft rubber liner was added to the treadmill so that animals' limbs would not be injured on the rough treadmill track.

Morris Water Maze.

The Morris Water Maze is a large pool (diameter = 165.1 cm, 81.28 cm high) that will be filled with water (54 cm deep; ~ 68°F/20° C). White, non-toxic tempura paint was added to the water to visually occlude the platform. The platform was placed in the pool and the surface of the platform was approximately five millimeters below the surface of the water. A large blue sheet was hung around the pool and visual cues at each cardinal direction (N, S, E, and W) were affixed to the sheet (See Figure 5). A camera was hung above the pool to capture video of each trial. Topscan (version 2.0) software was use to record and quantify variables in interest during MWM training.

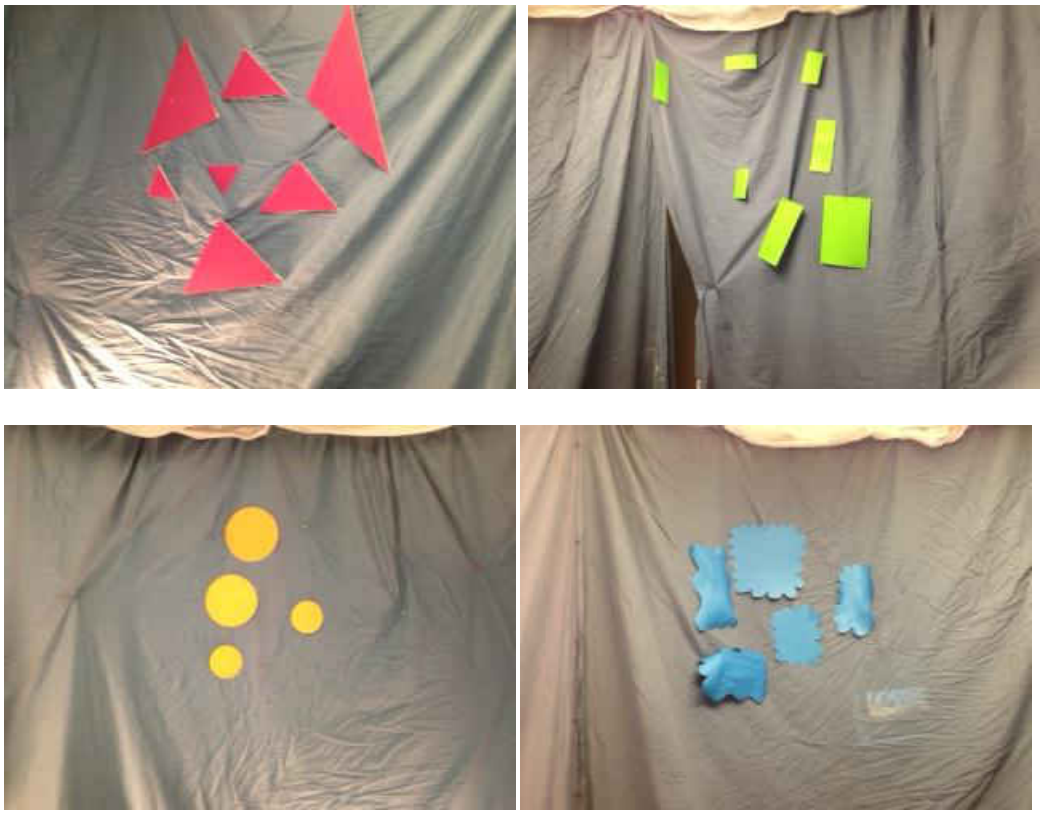


Figure 5. Spatial cues for the MWM. The cues were located on sheets at each of the four cardinal directions.

Procedure.

Voluntary exercise.

Animals were housed in the wheel cages for seven days. The number of revolutions each animal ran was recorded each day between the hours of 10 AM and 2 PM. On the seventh day, animals were removed from the wheel cages and placed in standard shoebox cages. Twenty-four hours after removal from the wheel cages, five of the 13 animals were sacrificed and the other eight animals began MWM training.

Forced exercise.

Animals in the forced exercise condition were exposed to the same six-day pre-exposure procedure as detailed in Experiment I (See Table 1). After pre-exposure and during the one week experimental period, FX animals had a total of five exercise

sessions, and two non-consecutive rest days. Animals ran at 0.5 mph (~15 meters per minute) for the entire 20 minute running session, rather than running at gradually faster speeds over the course of the first five minutes as in Experiment I [3]. Twenty-four hours after the last running session, five FX animals were sacrificed and the remaining eight underwent MWM training.

Drug and vehicle treatment.

Animals in the DMOG and VEH groups received injections each day for one week. DMOG (Frontier Scientific, Logan, UT) was diluted in dimethyl sulfoxide (DMSO) and further diluted to a 25% concentration in sterile saline. DMOG was injected intraperitoneally at a concentration of 50mg/kg, as described in the study by Ogle and colleagues (2012); injection volume ranged from approximately 0.3 to 0.5 mL per injection. A DMSO and saline solution was also prepared and injected in the VEH animals at the same concentration as the DMOG-treated animals.

Morris Water Maze.

After the culmination of the one week experimental period, eight animals from each group were trained in the MWM . Each animal started each trial with its nose facing toward the outside and against the wall of the pool. The starting location for each trial was determined randomly among the four quadrants which were defined by the four directions: North (N), South (S), East (E), and West (W). The animal was allowed to swim unhindered until it found the hidden platform, or until 60 seconds had elapsed. If the animal located the hidden platform it was allowed to remain on the platform for 10 seconds. If the animal failed to find the platform during the 60 seconds, it was placed on

[3] The newer treadmill used in Experiment II did not allow for speeds lower than 0.5 mph, so the speed ramping was not conducted in this experiment.

the platform and was allowed to remain there for 10 seconds. Following each trial, the animal was towel-dried and returned to its home cage to await the start of its next trial, which began after all other animals had undergone the same trial. Animals underwent four trials per day, one trial per starting location (N, S, E, W).

The MWM protocol utilized for this study assessed acquisition, reacquisition, and retention of spatial information. Before beginning actual MWM training, each animal underwent a single probe trial during which it swam for 60 seconds. No platform was placed in the pool for this first exposure. During actual training, for the first three days the hidden platform was located in the Northwest (NW) quadrant of the pool (acquisition phase). This phase of training assesses animals' learning speed. For days four through six, the location of the platform was changed to the Southeast (SE) quadrant (reacquisition phase). This phase of training allows for the assessment of whether or not the animal is able to assimilate its search strategy to find the platform upon relocation. After the sixth day of MWM training, animals were housed undisturbed in their home cages. One week after the final reacquisition training (day 13) day, animals underwent a single trial to test for retention. For this trial, the platform was removed. This probe trial allows for the analysis of memory retention as indicated by the amount of time the animal spent in the previously correct (southeast) quadrant.

Variables of interest in the MWM included: latency to find platform, time in correct quadrant, percent time in correct quadrant, correct quadrant entries, total quadrant entries, swim speed, and swim distance.

Tissue preparation.

Immunohistochemistry.

Animals were euthanized and tissue was prepared as described in Experiment I. Also as described in the first experiment, immunohistochemistry for HIF-1α was completed. The DAB procedure utilized for immunohistochemistry also allowed us to visualize blood vessels and quantify blood vessel density, so no counterstaining procedures were used.

Tissue imaging and quantification.

Images were taken in the same manner as described in Experiment I, and two images per region per slice were randomly selected for quantification. Also as in Experiment I and a random number generator was used to numerically code images for quantification. HIF-1α area fractions were quantified using the Image J-based thresholding and subtraction procedure described above.

The same images in which HIF-1α was quantified were also used for blood vessel density measurements. After performing the Image J HIF-1α thresholding procedure, the original image was opened once again, and the region of interest outlined. A point grid was then superimposed upon the image. Each image contained a total of 80 possible points. If the region of interest did not occupy the entire image, the number of points within the region of interest was counted. Finally, the number of points falling on or within blood vessels was counted to give a ratio of the points falling on or within blood vessels to the total points within the region of interest. Blood vessel density was quantified as follows:

$$\text{Blood vessel density} = \left(\frac{\text{\# points on or within BV}}{\text{total points ROI}} \right) \text{x} 100$$

Statistical analyses.

Anatomy.

To determine if there were significant differences among groups in HIF-1α expression and blood vessel density, two multivariate ANOVAs were conducted, one for area fraction, and one for blood vessel density. The three dorsal hippocampal regions of interest (CA1, CA2/3, and the DG). If the multivariate ANOVAs detected significant differences, the subsequent univariate tests were examined, and the least significant difference (LSD) post-hoc test was used to determine where any specific group differences occurred.

Morris Water Maze.

MWM data was analyzed using a mixed model repeated measures ANOVAs with day as the within-subjects repeated measure and group as the between-subjects fixed factor where appropriate. Analyses for each phase of training were conducted separately. Variables of interest during the acquisition phase included latency to find the hidden platform, percent time in correct quadrant, correct quadrant entries, trials to criterion, total quadrant entries, swim distance, and swim speed. For reacquisition, we analyzed the percent time spend in the previously correct quadrant, and previously correct quadrant entries, in addition to the variables analyzed during acquisition. Finally, the variables of interest in the retention test included percent time spent in the acquisition quadrant, and percent time spent in the reacquisition quadrant. Other measures such as percent change scores, were analyzed using univariate ANOVAs with group as the independent variable.

Post-hoc tests using the LSD correction were used to determine specific group differences if the omnibus ANOVA indicated significant differences.

Results

Experiment I

Tissue from two animals (one from group IC2, one from group ICLT) was not used in the analysis due to poor immunohistochemical staining, leaving a total of 98 animals in the analysis.

An analysis for the presence of outliers revealed four outliers in area CA1 (one animal from group IC0, two animals from group IC6, and one animal from group EX2), leaving data points from 94 animals in the CA1 analyses. Four outliers were also identified in region CA2/3 (one animal from group IC0, two animals from group IC2, and one animal from group EXLT), leaving data points from 94 animals in the CA2/3 analyses. Finally, two outliers were identified in the DG (both from group IC2), leaving 96 data points for the DG analyses.

The three-way ANOVA indicated a significant time x region x activity interaction ($F_{(8,254)} = 2.024$, $p < 0.05$; see Figure 6), suggesting that HIF-1α expression is dependent upon the interplay of the three factors. The time x activity interaction was also statistically significant ($F_{(4, 254)} = 5.353$, $p < 0.001$), and there was a significant main effect of time ($F_{(4,254)} = 4.564$, $p < 0.01$) and of region ($F_{(2,254)} = 17.713$, $p < 0.001$). However, there was not a significant main effect of activity ($F_{(1,254)} = 0.435$, $p = 0.510$), which is likely due to the high CA1 area fraction in the long-term inactive group.

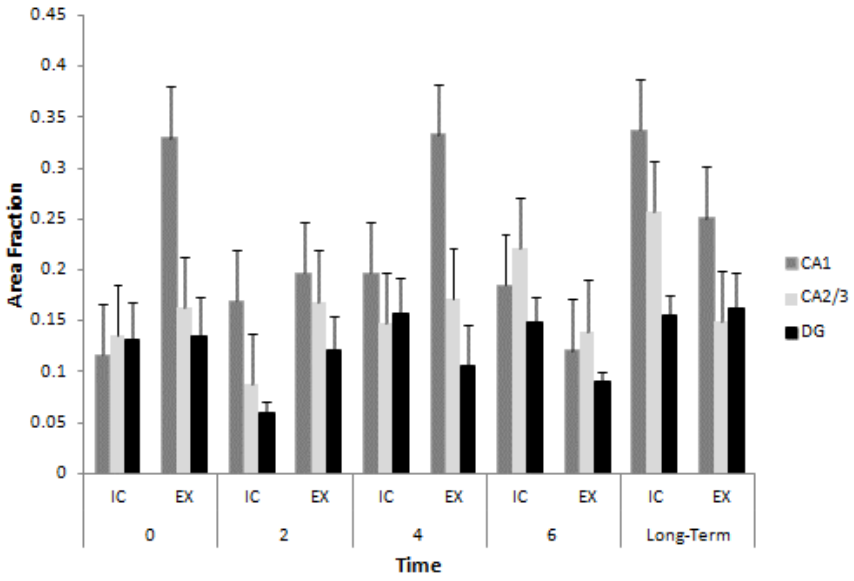


Figure 6. HIF-1α expression by region. The three-way interaction among activity, time, and region was statistically significant ($p < 0.05$).

EX animals.

CA1.

Among exercising animals, EX0 animals had significantly greater HIF-1α area fraction in area CA1 compared to EX2 ($M = 0.197$; $p = 0.011$) and EX6 animals ($M = 0.121$; $p < 0.001$). In addition, EX4 animals also had significantly greater HIF-1α area fraction in CA1 compared to EX2 ($p < 0.01$) and EX6 animals ($p < 0.001$; see Figure 7). Animals undergoing chronic exercise (EXLT) had significantly greater HIF-1α expression in CA1 compared to EX6 animals ($p = 0.010$), but did not differ significantly from EX0 ($p = 0.114$), EX2 ($p = 0.306$), or EX4 ($p = 0.104$) animals. See Figure 8 for representative immunohistochemistry images.

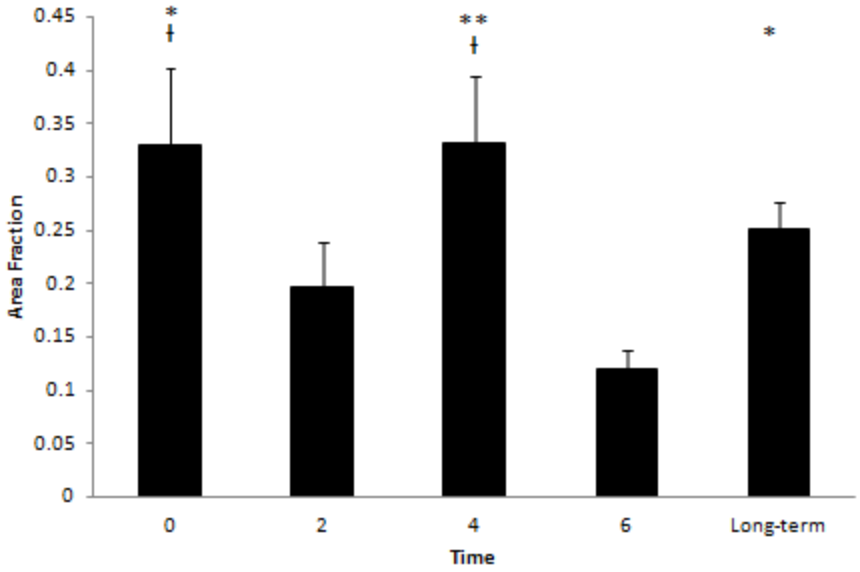


Figure 7. CA1 HIF-1α area fraction in exercising animals. EX0 vs. EX2 (*$p < 0.05$), EX0 vs. EX6 ($^{\dagger}p < 0.001$), EX4 vs. EX2 (**$p < 0.01$), EX4 vs. EX6 ($^{\dagger}p < 0.001$), and EXLT vs. EX6 (*$p < 0.05$).

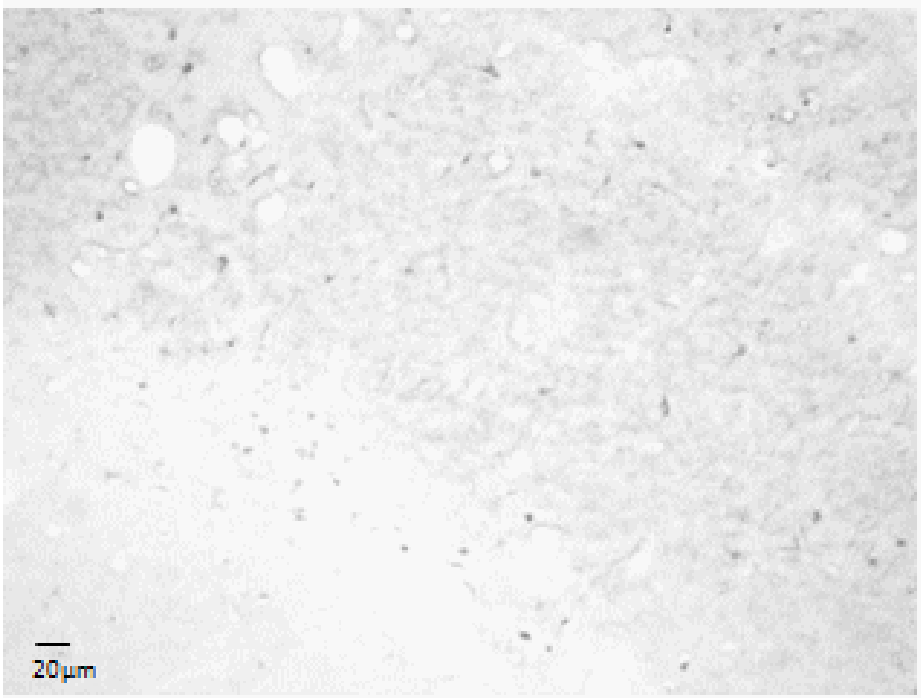


Figure 8. HIF-1α immunohistochemistry, 400x magnificantion.

CA2/3 and DG.

As illustrated in Figure 9 and Table 2, there were no significant differences in HIF-1α expression in area CA2/3 or the DG at any time point among exercising animals (data not presented).

Table 2.

Mean (SEM) HIF-1α Area Fraction in Exercising and Inactive Animals

	Time (hours)									
	0		2		4		6		Long-Term	
Activity	IC	EX	IC	EX	IC	EX	IC	EX	IC	EX
Region										
CA1	0.117 (0.024)	0.330 (0.073)	0.170 (0.026)	0.197 (0.043)	0.197 (0.029)	0.333 (0.062)	0.185 (0.026)	0.121 (0.017)	0.337 (0.064)	0.251 (0.026)
CA2/3	0.136 (0.022)	0.163 (0.032)	0.088 (0.009)	0.169 (0.035)	0.147 (0.030)	0.172 (0.032)	0.221 (0.051)	0.140 (0.015)	0.257 (0.046)	0.150 (0.018)
DG	0.132 (0.037)	0.135 (0.038)	0.059 (0.012)	0.121 (0.034)	0.157 (0.036)	0.106 (0.040)	0.149 (0.025)	0.091 (0.025)	0.155 (0.021)	0.162 (0.036)

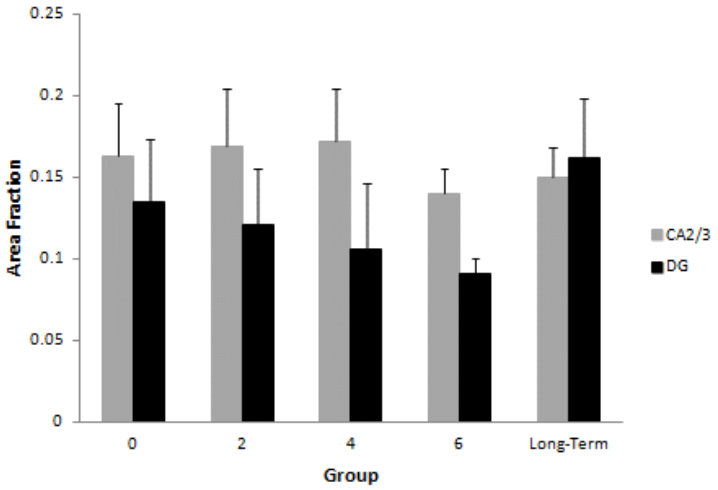


Figure 9. Neither CA2/3 nor DG HIF-1α expression differed significantly among groups of exercising animals.

IC animals.

CA1.

In area CA1, no comparisons among short-term inactive animals reached or approached statistical significance. However, long-term inactive (ICLT) animals had significantly greater HIF-1α expression compared to all short-term IC groups (vs. IC0, $p < 0.001$; vs. IC2, $p < 0.01$; vs. IC4, $p < 0.01$; and vs. IC6, $p < 0.01$; See Figure 10). Please refer to Table 2 for mean and standard error HIF-1α area fractions.

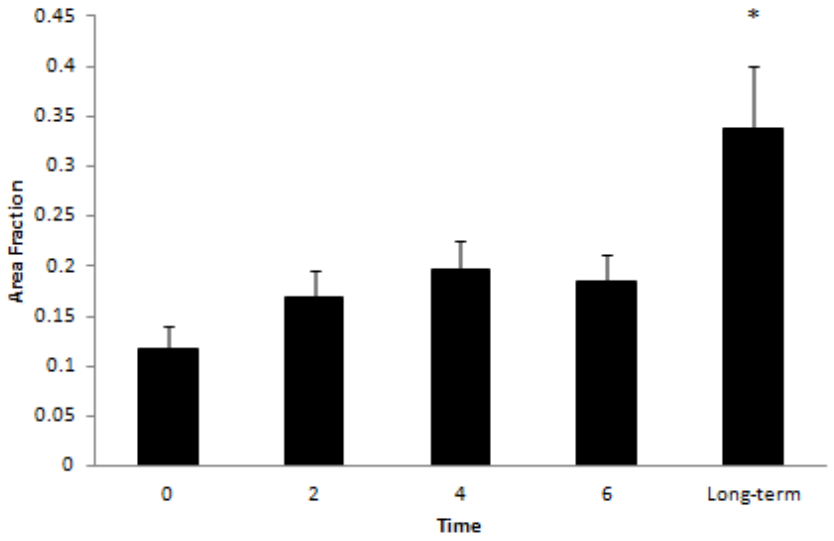


Figure 10. CA1 area fraction in IC animals. ICLT animals had significantly greater HIF-1α area fraction compared to all short-term IC groups

CA2/3 and DG.

Among inactive animals expression of HIF-1α in area CA2/3 did differ among groups. Specifically, in the short-term groups, HIF-1α area fraction was significantly greater in IC6 animals ($M = 0.221$) compared to IC2 animals ($M = 0.088$, $p = 0.018$). Additionally, ICLT ($M = 0.257$) animals had significantly higher area fraction in CA2/3 compared to IC0 ($M = 0.136$, $p = 0.023$), IC2 ($p = 0.003$), and IC4 animals ($M = 0.147$, p

= 0.034). There were no significant differences among any groups of inactive animals in the DG (see Figure 11).

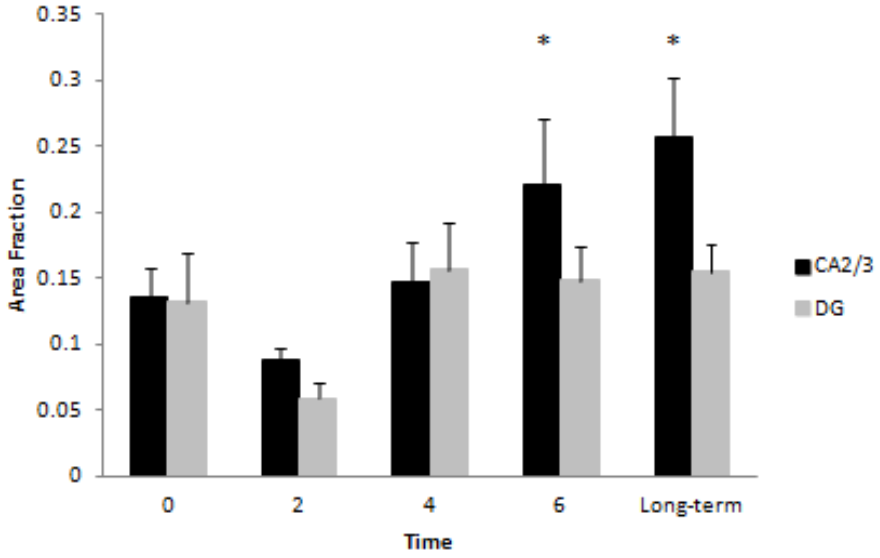


Figure 11. In CA2/3, ICLT animals had greater HIF-1α expression compared to IC0, IC2, and IC4 animals; IC6 animals had greater expression compared to IC2 animals. DG HIF-1α expression was similar in all inactive groups.

EX vs. IC Comparisons.

CA1.

Post-hoc tests indicated that exercising animals sacrificed at zero and four hours (M = 0.330 and M = 0.333, respectively) after treadmill exercise had significantly increased HIF-1α expression in area CA1 compared to IC0 (M = 0.117, $p < 0.001$) and IC4 animals (M = 0.197, $p < 0.01$; see Figure 12). There were no significant differences between inactive and exercising animals at two hours (EX2, M = 0.197; IC2, M = 0.170), at six hours (EX6, M = 0.121; IC6, M = 0.185), or at the long-term time point (EXLT, M = 0.251; ICLT, M = 0.337).

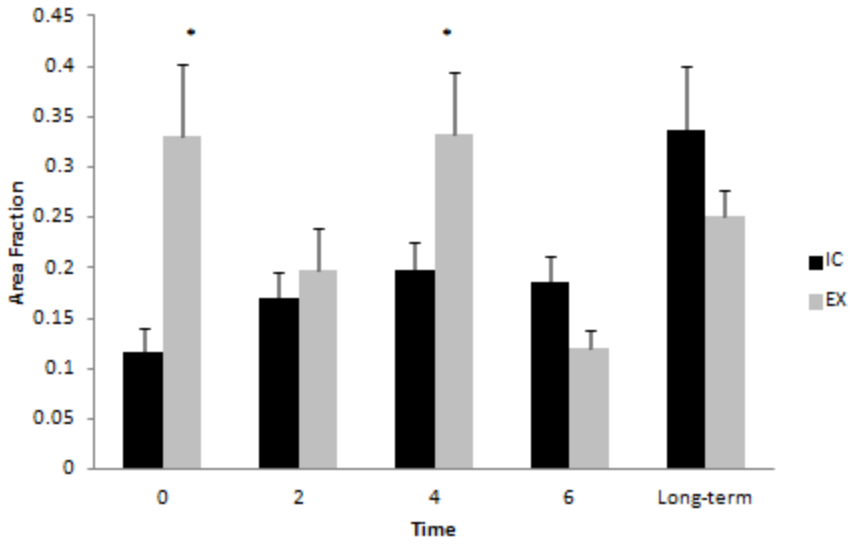


Figure 12. In CA1, differences between IC and EX animals were significantly different only at zero and four hours following treadmill exercise or exposure.

CA2/3 and DG.

In area CA2/3, the comparison between ICLT (M = 0.257) and EXLT animals (M = 0.150) was the only comparison to reach statistical significance, with ICLT animals having significantly greater HIF-1α expression than EXLT animals (p = 0.045; see Figure 13). HIF-1α expression in the DG was not significantly different among inactive and exercising animals at any time point (data not presented).

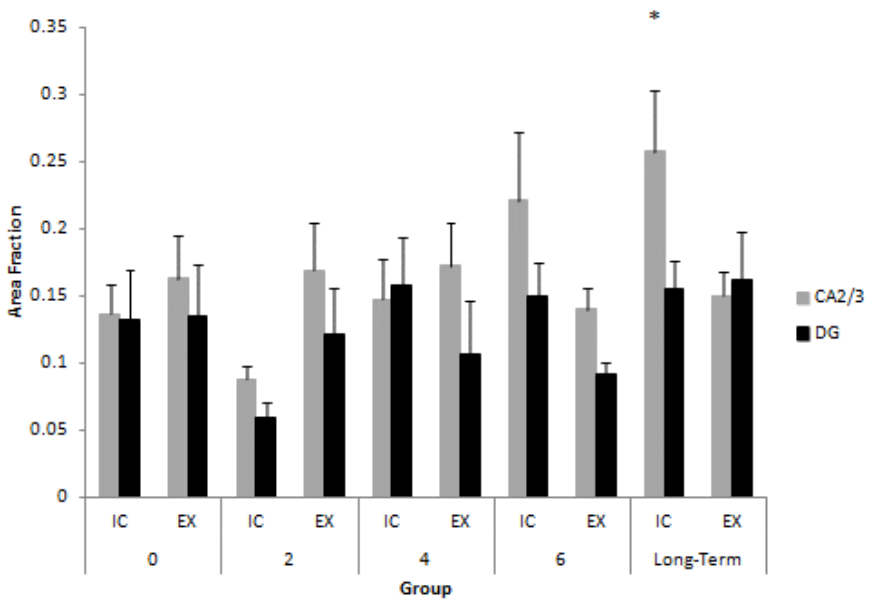


Figure 13. ICLT animals had significantly greater HIF-1α area fraction compared to EXLT animals. No other comparisons between EX and IC animals in CA2/3 or in DG reached statistical significance.

Experiment II

In Experiment I we found that acute exercise causes an upregulation of HIF-1α in area CA1 of the hippocampus. Because exercise is known to cause significant enhancements in cognitive performance (Gomez-Pinilla, et al., 2008; Griesbach et al., 2009; Kerr et al., 2010; van Praag et al., 1999; Vaynman et al., 2004references), and based on the findings of Adamcio et al. (2010) who found that treatment with a HIF-1α agonist caused significant improvements in contextual memory during fear conditioning, we hypothesized that HIF-1α may be involved in exercise-induced cognitive facilitation. VEGF is one of the target genes of HIF-1α and is involved in angiogenesis during development (reviewed in Lee, et al., 2009), in pathological conditions (reviewed in Folkman, 1995), and following engagement in exercise (Black et al., 1990; Ding et al., 2006a; Isaacs et al., 1992; Kleim et al., 2002; Sikorski et al., 2008; Swain et al., 2003; Van der Borght et al., 2009). The role of angiogenesis in exercise-induced cognitive

facilitation was investigated by Kerr and colleagues (2010) who showed that inhibition of angiogenesis led to deficits in acquisition during the MWM. Therefore, it is possible that after the commencement of exercise, increases in HIF-1α expression cause angiogenesis which then promotes improved behavioral performance in learning and memory tasks.

The purpose of Experiment II was to determine if increased HIF-1α expression and/or increased capillary density would affect learning and memory performance in the MWM. We also sought to compare and contrast the expression of HIF-1α, angiogenesis, and cognitive performance of drug-treated animals to that of exercising animals. If HIF-1α and angiogenesis mediate behavioral facilitation, we would expect similar anatomical findings in drug-treated and exercising animals, and similar performances in the MWM.

To manipulate the expression of HIF-1α we administered a HIF-1α agonist. Dimethyloxalylglycine (DMOG) is a prolyl hydroxylase inhibitor, and has been shown to increase the expression of HIF-1α 12 and 24 hours after a single intraperitoneal injection (Ogle et al., 2012). Two different exercise paradigms were also used to determine if the pattern of exercise, namely forced (treadmill) or voluntary exercise, would result in different anatomical findings and/or different behavioral performances.

Anatomy.

Immunohistochemisty was conducted to determine the level of expression and the local expression of HIF-1α in three regions of the hippocampus: CA1, CA2/3, and the DG. Additionally, the colorimetric DAB staining used during immunohistochemistry allowed us to also quantify blood vessel density.

HIF-1α expression.

The multivariate ANOVAs indicated that there were significant differences among groups in HIF-1α area fraction ($F_{(12,47)} = 3.728$, $p < 0.01$; see Figure 14). Univariate tests also indicated significant differences in all three hippocampal regions (data shown below).

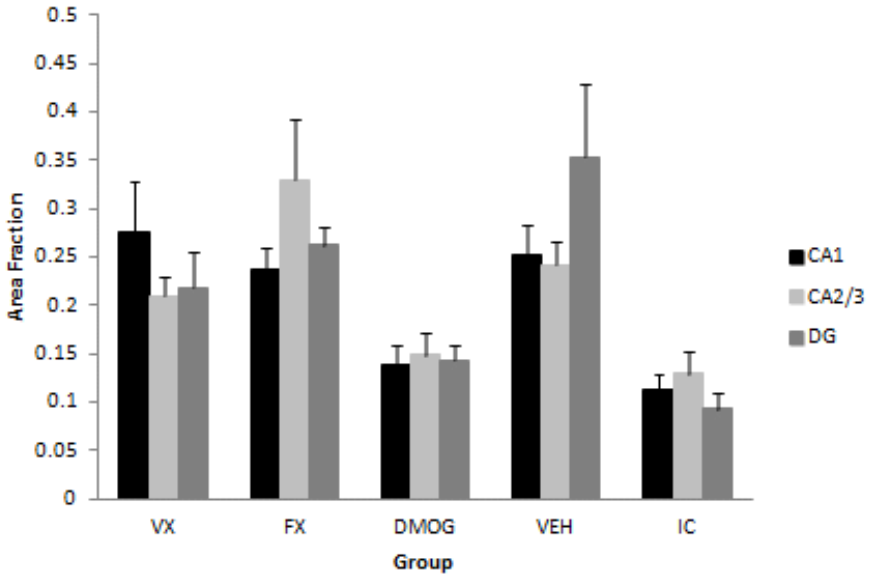


Figure 14. HIF-1α expression was different among groups in all three hippocampal regions ($p < 0.01$).

CA1.

As shown in Figure 15, univariate tests showed that HIF-1α area fraction was significantly different among the four groups in area CA1 ($F_{(4,20)} = 5.217$, $p = 0.005$). Least significant difference tests showed that CA1 HIF-1α expression was significantly greater in VX animals ($M = 0.275$) compared to DMOG-treated animals ($M = 0.138$; $p < 0.01$) and IC animals ($M = 0.112$; $p < 0.01$). FX ($M = 0.237$) animals also had significantly increased HIF-1α expression in CA1 compared to both DMOG ($p < 0.05$) and IC animals ($p < 0.05$). Surprisingly, VEH-treated animals ($M = 0.252$) also had

significantly greater HIF-1α expression than DMOG-treated animals ($p = 0.02$) and IC animals ($p < 0.01$).

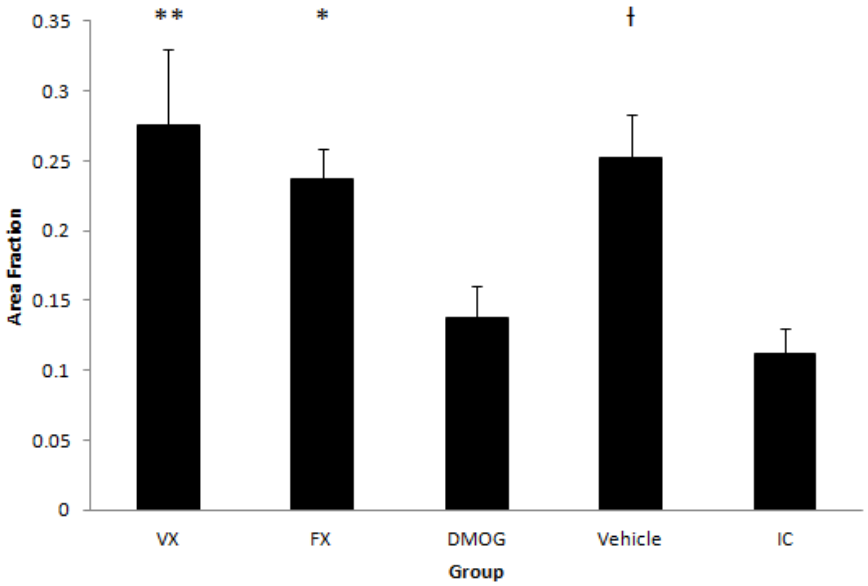


Figure 15. In area CA1, VX, FX, and VEH animals had significantly greater HIF-1α area fraction compared to DMOG and IC animals. ** vs. DMOG, $p < 0.01$; vs IC, $p < 0.01$. * vs. DMOG, $p < 0.05$; vs. IC, $p = 0.012$. ǂ vs. DMOG, $p = 0.02$; vs. IC, $p < 0.01$.

Table 3.

Mean (SEM) HIF-1α area fraction by hippocampal region.

	Hippocampal Region		
Group	CA1	CA2/3	DG
VX	0.275 (0.053)	0.210 (0.020)	0.218 (0.037)
FX	0.237 (0.022)	0.330 (0.063)	0.263 (0.019)
DMOG	0.138 (0.022)	0.149 (0.022)	0.143 (0.016)
VEH	0.252 (.031)	0.242 (0.025)	0.354 (0.075)
IC	0.112 (0.018)	0.130 (0.023)	0.093 (0.017)

CA2/3.

Univariate tests also showed significant differences among groups in area CA2/3 ($F_{(4,20)} = 5.234$, $p < 0.01$). Post-hoc analysis (LSD) indicated that in area CA2/3, FX

animals had significantly increased HIF-1α expression compared to VX, (p = 0.025), DMOG (p = 0.002), and IC (p = 0.001) animals, but FX animals did not differ significantly from VEH animals (p = 0.091; see Figure 16). VX animals had similar HIF-1α expression compared to DMOG (p = 0.231), VEH (p = 0.526), and IC (p = 0.118) animals. Finally, VEH animals had significantly increased area fraction compared to IC (p = 0.034) animals.

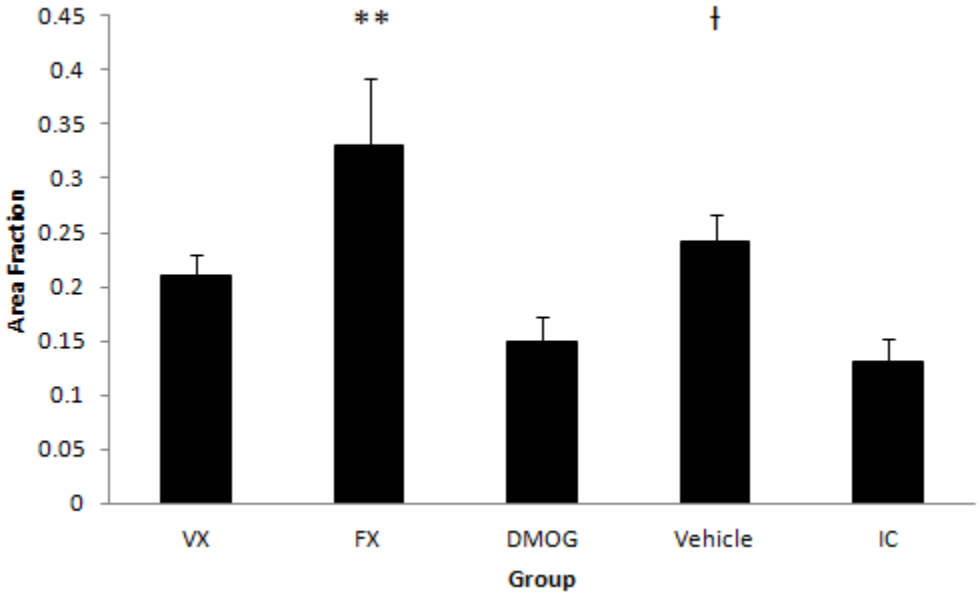


Figure 16. FX animals had the greatest HIF-1α expression in area CA2/3. ** vs. VX, p = 0.025; vs. DMOG, p = 0.002; vs. IC, p = 0.001. ŧ vs. IC, p = 0.034.

DG.

Results for the DG were similar to CA1 and CA2/3 results in that univariate tests indicated a significant difference among treatment conditions in HIF-1α area fraction ($F_{(4,20)}$ = 6.600, p = 0.001). In the DG, VEH-treated animals had the greatest expression of HIF-1α, with significantly increased expression compared to VX (M = 0.218; p = 0.026), DMOG (M = 0.143; p = 0.001), and IC animals (M = 0.093; p < 0.001), but not

compared to FX animals ($M = 0.263$, $p = 0.121$; see Figure 17). VX animals did have significantly greater area fraction compared to IC animals ($p = 0.037$), and compared to both IC and DMOG animals, FX animals had significantly increased HIF-1α expression ($p = 0.007$ and $p = 0.046$, respectively).

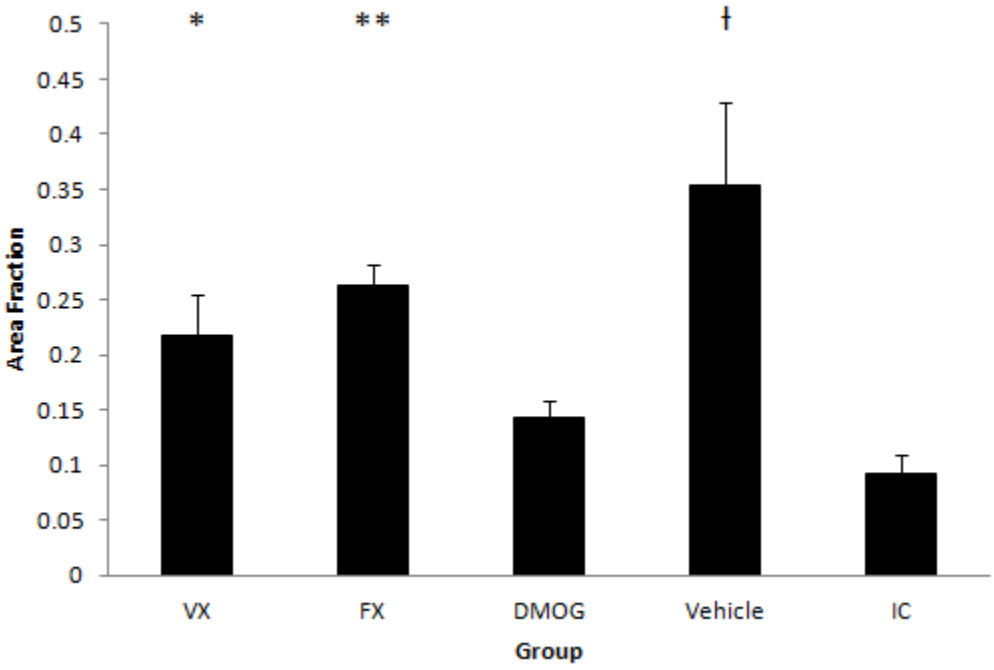


Figure 17. VEH animals had significantly greater DG HIF-1α area fraction compared to all groups (ɬ vs. VX, p = 0.026; vs. DMOG, p = 0.001; vs. IC, p < 0.001) with the exception of the FX group. VX and FX animals showed higher expression compared to IC (* p = 0.037), and DMOG and IC groups (** p = 0.046; p = 0.007), respectively.

Blood vessel density.

The multivariate ANOVA also indicated that there were significant differences among groups in blood vessel density ($F_{(12,47)} = 1.998$, $p = 0.045$). Follow-up univariate tests as well as post-hocs comparisons (LSD) were found to be significant in CA1 and the DG, but not in area CA2/3 (see Figure 18). Please see Table 4 for mean and standard error blood vessel density measures for each hippocampal region.

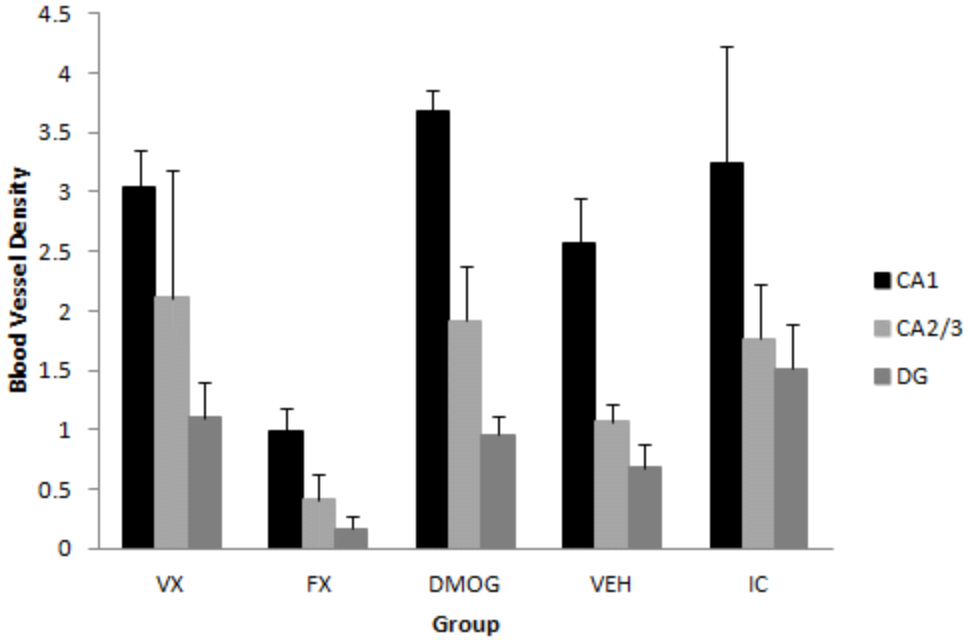


Figure 18. Blood vessel density is affected by region and group. Significant differences were found among groups in CA1 and the DG, but not in CA2/3.

Table 4.

Mean (SEM) Blood Vessel Density by Hippocampal Region.

	Hippocampal Region		
Group	CA1	CA2/3	DG
VX	3.039 (0.317)	2.112 (1.077)	1.099 (0.295)
FX	0.983 (0.207)	0.415 (0.216)	0.167 (0.108)
DMOG	3.680 (0.182)	1.915 (0.456)	0.961 (0.152)
VEH	2.564 (0.390)	1.067 (0.148)	0.679 (0.196)
IC	3.240 (0..994)	1.768 (0.465)	1.514 (0.371)

CA1.

The univariate ANOVA indicated a significant difference among groups in CA1 blood vessel density ($F_{(4,20)} = 4.113$, $p = 0.014$). VX ($M = 3.039$), DMOG ($M = 3.680$), and IC ($M = 3.240$) groups had the greatest blood vessel densities in area CA1 (see

Figure 19). Post-hoc tests using the LSD correction showed that all groups had significantly higher blood vessel density compared to FX animals ($M = 0.983$; vs. VX animals, $p = 0.010$; vs. DMOG animals, $p = 0.001$; vs. VEH animals, $p = 0.042$; vs. IC animals, $p = 0.006$). No other comparisons reached or approached significance.

Given the high degree of variability in the IC group in particular, we performed an outlier analysis on CA1 blood vessel density. In SPSS, outliers are defined as values that fall outside of one and a half box lengths of the box edges (Pallant, 2004). SPSS identified three outliers in area CA1: one animal each from the FX, DMOG, and IC groups. The multivariate ANOVA excluding outliers from area CA1 indicated significant differences among groups ($F_{(12,38)} = 2.253$, $p < 0.027$). The follow-up univariate test showed a significant difference in blood vessel density among the five groups of animals ($F_{(4,17)} = 6.056$, $p < 0.003$). Post-hoc comparisons using the LSD correction indicated that DMOG animals had significantly greater blood vessel density compared to FX ($p < 0.001$) and IC ($p < 0.05$) animals, and a trend toward significantly greater capillarity compared to VEH animals ($p = 0.068$). Furthermore, FX animals had significantly lower blood vessel density compared to animals in the VX ($p < 0.01$), VEH ($p = 0.011$), and IC ($p < 0.05$) groups. These results are depicted in *Figure 20*.

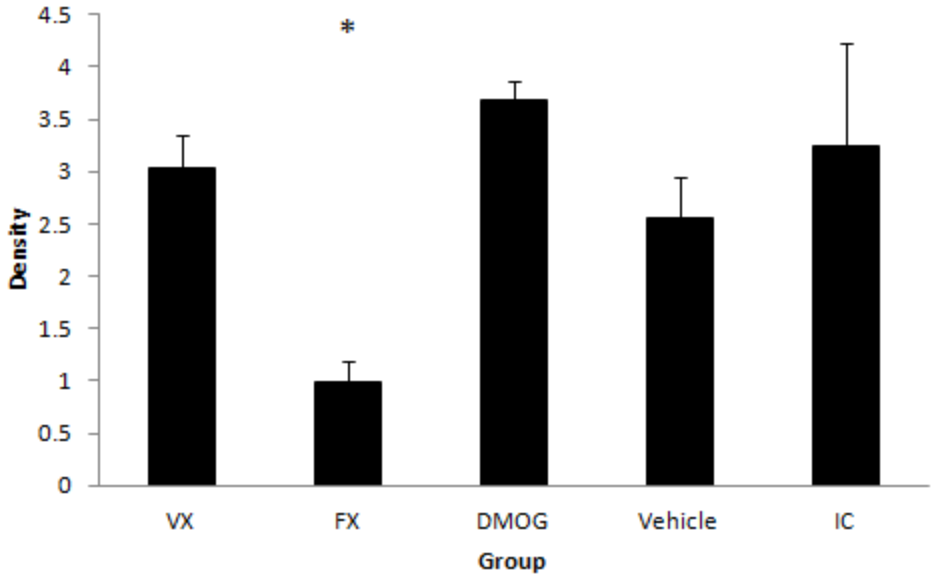


Figure 19. CA1 blood vessel density. VX, DMOG, and IC animals had the greatest blood vessel density in CA1. FX animals had significant lower blood vessel density compared to all other groups of animals. *$p <$ 0.05 for all comparisons.

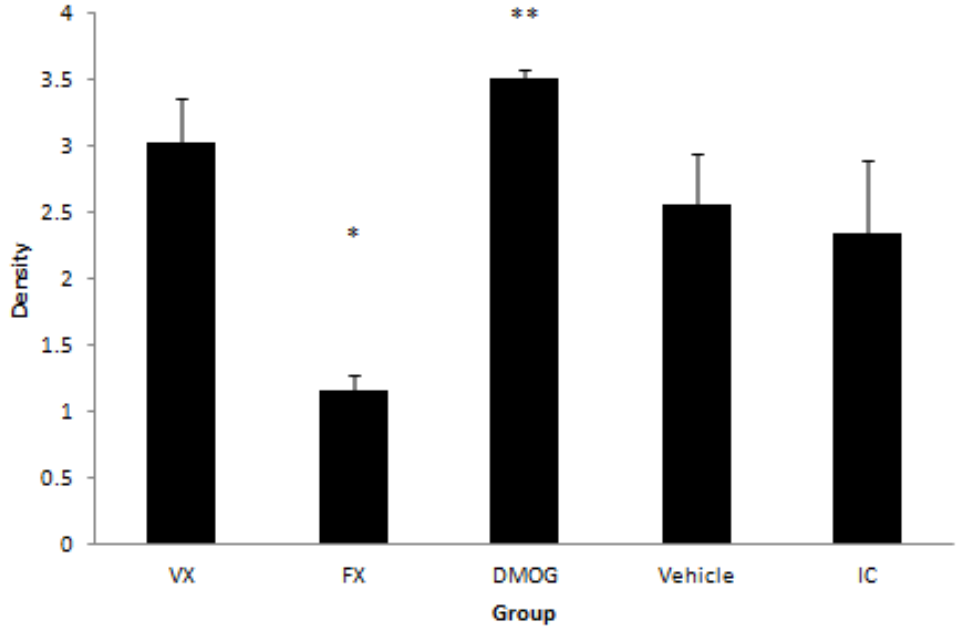


Figure 20. CA1 blood vessel density excluding outliers. FX animals had significantly lower blood vessel density compared to all other groups of animals ($p < 0.05$). DMOG animals had significantly greater blood vessel density compared to IC ($p < 0.05$) and trended toward significantly increased density compared to VEH animals ($p = 0.068$)

CA2/3.

Similar to area CA1, in area CA2/3 VX (M = 2.112), DMOG (M = 1.915), and IC (M = 1.768) animals had the greatest blood vessel densities. However, the univariate ANOVA for CA2/3 blood vessel density did not show significant differences among groups ($F_{(4,20)}$ = 1.492, p = 0.242; see Figure 21).

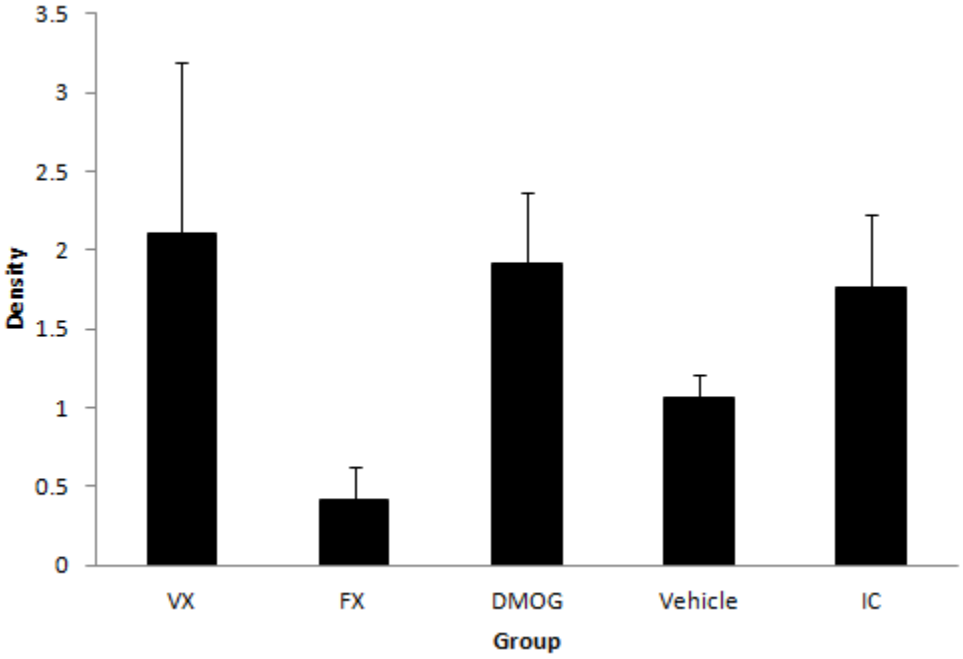


Figure 21. CA2/3 blood vessel density. No significant differences were found among the groups in CA2/3 blood vessel density, but similar to densities in CA1, VX, DMOG, and IC animals did have the greatest densities.

DG.

As illustrated in Figure 22, there were significant differences among groups in DG blood vessel density ($F_{(4,20)}$ = 4.208, p = 0.012). VX (M = 1.099) and IC (M = 1.514) animals had the greatest blood vessel densities and differed significantly from FX animals (M = 0.167; p = 0.014 and p = 0.001, respectively). FX animals also had significantly fewer blood vessels compared to DMOG animals (M = 0.961, p = 0.032).

Finally, IC animals also had significantly greater blood vessel density in DG compared to VEH animals ($M = 0.679$, $p = 0.025$).

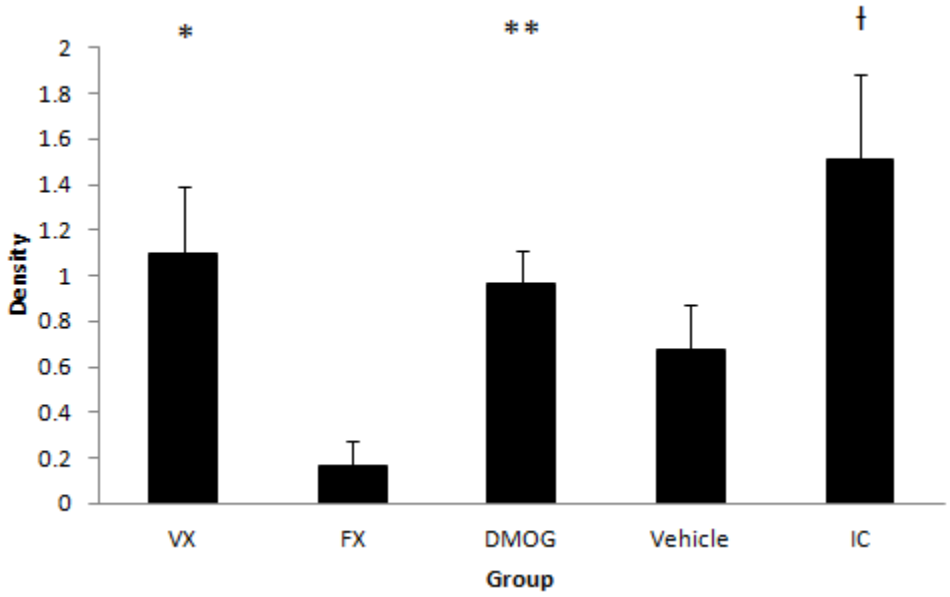


Figure 22. DG blood vessel density. VX and IC animals had highest density measurements which were significantly higher than FX animals (* $p = 0.014$, † $p = 0.001$, repsectively). DMOG animals also had greater blood vessel density than FX animals (** $p = 0.032$). IC density was also significantly greater than VEH († vs. VEH, $p = 0.025$).

Morris Water Maze (MWM).

The MWM paradigm used for this experiment consisted of four trials per day, with each trial beginning at a different location around the perimeter of the pool. We also employed two different learning phases: acquisition (days one through three) and reacquisition (days four and five). Finally, one week after the last reacquisition day (day 13) animals were assessed for retention in a single probe trial in the absence of the escape platform.

Eight of the 13 animals from each group were trained in the MWM, with the exception of the FX group which is comprised of seven animals in all analyses; one animal in the FX group refused to run on the treadmill and was therefore removed from the study.

Acquisition phase.

Latency to find the hidden platform.

Latency to find the hidden platform is a commonly used measure to analyze MWM performance; successful learning is reflected in shorter latencies to find the hidden platform. As illustrated in Figure 23, VX, DMOG, and IC animals performed the best during the acquisition phase of MWM training. Within groups, there was a significant effect of day ($F_{(2,33)} = 19.593$, $p < 0.001$). DMOG, VX, and IC animals found the hidden platform more quickly than the VEH and FX animals on all three acquisition days (see Table 5), but there was not a significant day by group interaction ($F_{(8,68)} = 0.861$, $p = 0.572$).

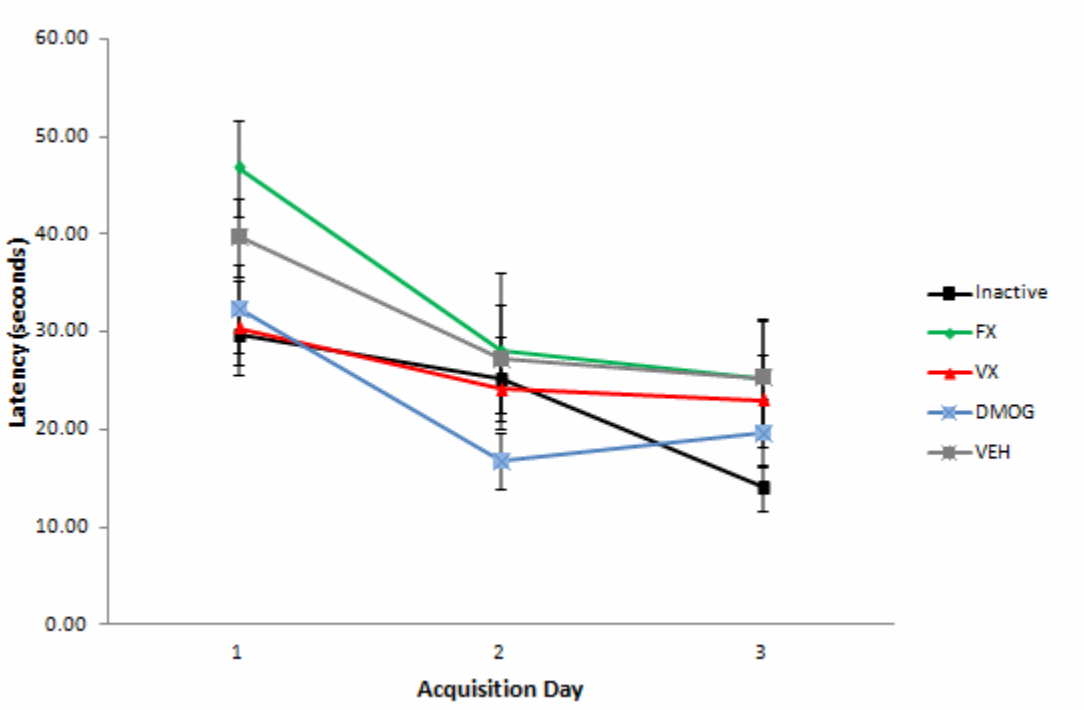


Figure 23. Acquisition phase -latency to find the hidden platform. DMOG, VX, and IC animals were the fastest to find the platform on all three acquisition days, but there were no significant differences in latencies among the groups during acquisition ($p = 0.572$). There was however, a significant effect of day among all 5 groups ($p < 0.001$).

Table 5.

Mean (SEM) Latency to Find the Hidden Platform During MWM Acquisition.

Group	Acquisition Day		
	1	2	3
IC	29.75 (3.13)	25.21 (4.25)	14.04 (2.35)
FX	46.82 (4.91)	28.03 (7.98)	25.27 (6.06)
VX	30.39 (4.78)	24.13 (2.50)	23.01 (4.70)
DMOG	32.43 (4.51)	16.79 (2.80)	19.64 (3.42)
VEH	39.72 (3.97)	27.16 (5.54)	25.27 (5.86)

Percent change in latency to find hidden platform.

Another way that learning can be assessed in the MWM is by calculating a percent change in latency to find the hidden platform. This measure can be interpreted as the rate of animal learning and is calculated with the following formula (AD = acquisition day):

$$\left[\frac{\text{AD3 Latency - AD1 Latency}}{\text{AD1 Latency}}\right] \text{x} 100$$

A univariate analysis of variance indicated that there was not a significant group by acquisition day interaction ($F_{(4,34)} = 0.887$, $p = 0.482$). Figure 24 shows that IC and FX animals improved the most in escape latency, whereas VX and DMOG animals did not have large changes in time to reach the platform. This could be due to the fact that both VX and DMOG animals found the platform fairly quickly across trials on day 1 ($M = 30.39$ and $M = 32.43$, respectively), leaving little room for improvement. To investigate the possibility and VX, DMOG, and IC animals learn the task faster, a univariate ANOVA was used to analyze latency to find the platform on acquisition day one alone. This analysis yielded a significant difference among groups in latency to find

the platform across the four trials in acquisition day one ($F_{(4,34)} = 2.779$, $p < 0.05$). Post-hoc tests using the LSD correction showed that FX animals ($M = 46.82$) took significantly longer to find the platform compared to IC ($M = 29.75$, $p < 0.01$), VX ($M = 30.39$, $p = 0.012$), and DMOG ($M = 32.43$, $p = 0.026$) animals on acquisition day one (see Figure 25).

The superior performance of the IC animals throughout the acquisition phase was unexpected, and the greater degree of change in latency to find the platform in FX and VEH groups is due to the fact that these animals took a long time to find the platform on day one. In summary, the percent change scores demonstrate that all animals acquired the task, but the rate of acquisition was highly variable among groups.

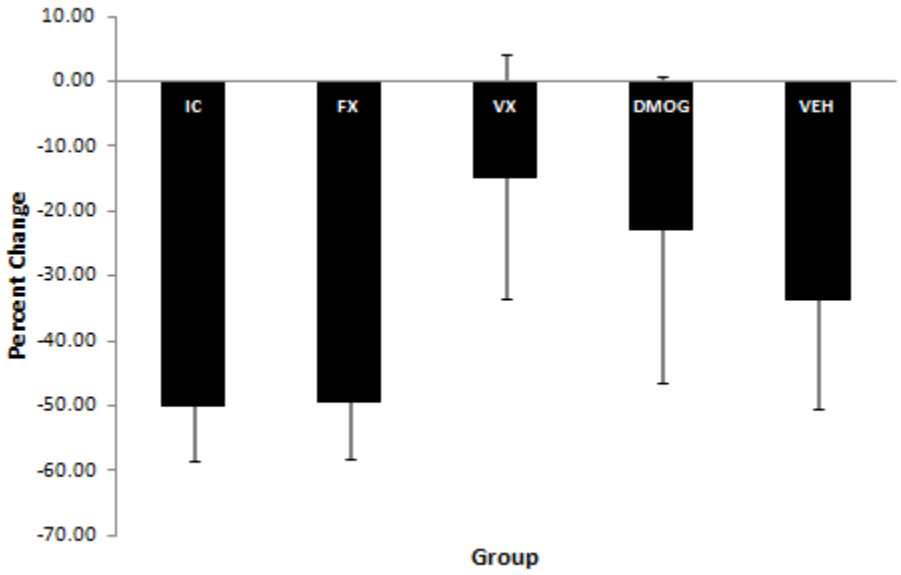


Figure 24. Acquisition phase percent change in latency to find the hidden platform. IC and FX animals demonstrated the most improvement in time to escape.

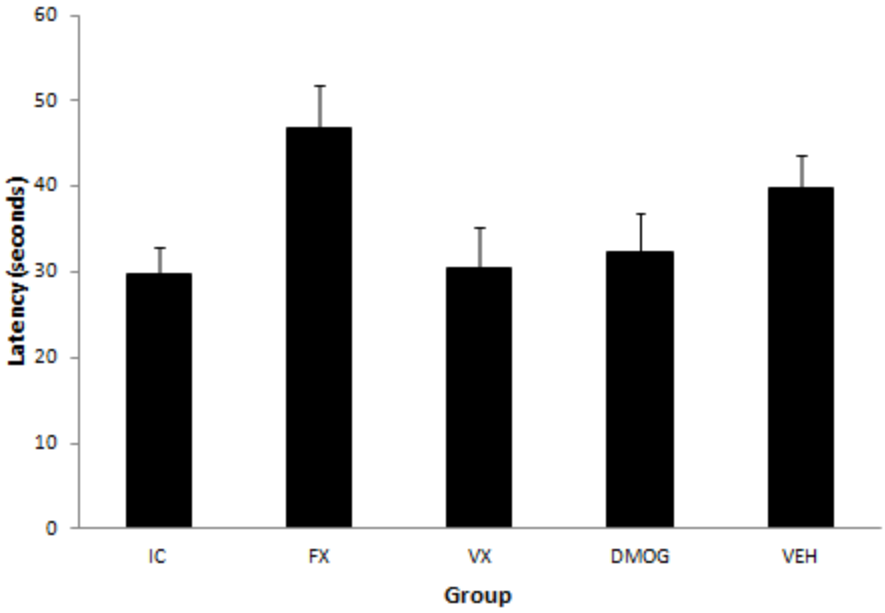


Figure 25. Acquisition day 1 – average latency to find the hidden platform. FX animals took significantly longer to find the hidden platform on day 1 compared to IC, VX, and DMOG animals ($p < 0.05$).

Percent success.

The proportion of successful trials can also be used as a measure of task acquisition, so we analyzed the percent success across the four trials for each of the three acquisition days. On the first day of acquisition, FX ($M = 46.43\%$) and VEH ($M = 50.00\%$) were the least successful in finding the hidden platform, whereas VX ($M = 71.88\%$), DMOG ($M = 65.63\%$), and IC ($M = 81.25\%$) were more successful at escaping within the allotted 60 seconds (see Figure 26 and Table 6). Statistical analyses, however, did not indicate a significant difference among groups across acquisition days in trial success ($F_{(8,68)} = 1.562$, $p = 0.153$). Again, this null finding could be due to the fact that all animals improved their performance from acquisition day one to acquisition day three. In fact, by day three, all groups of animals were finding the platform over 80% of the time. This improvement in performance in all groups is possibly washing out any significant effect in percent success among groups across the three acquisition days.

To test the hypothesis that certain group of animals, namely the exercising and drug-treated animals, will learn the task faster than others, and to further explore the proportion of successful trials among groups, a univariate analysis of variance was conducted on percent success scores on day one alone. This analysis revealed a trend toward significant group differences in the percent of successful trials on the first day of training ($F_{(4,34)} = 2.301$, $p = 0.079$). Follow-up tests using the LSD correction showed that on day one IC animals were significantly more successful in finding the hidden platform compared to VEH ($p = 0.026$) and FX ($p = 0.017$) animals. VX animals were marginally more successful in finding the platform on the first day of training compared to FX animals ($p = 0.075$).

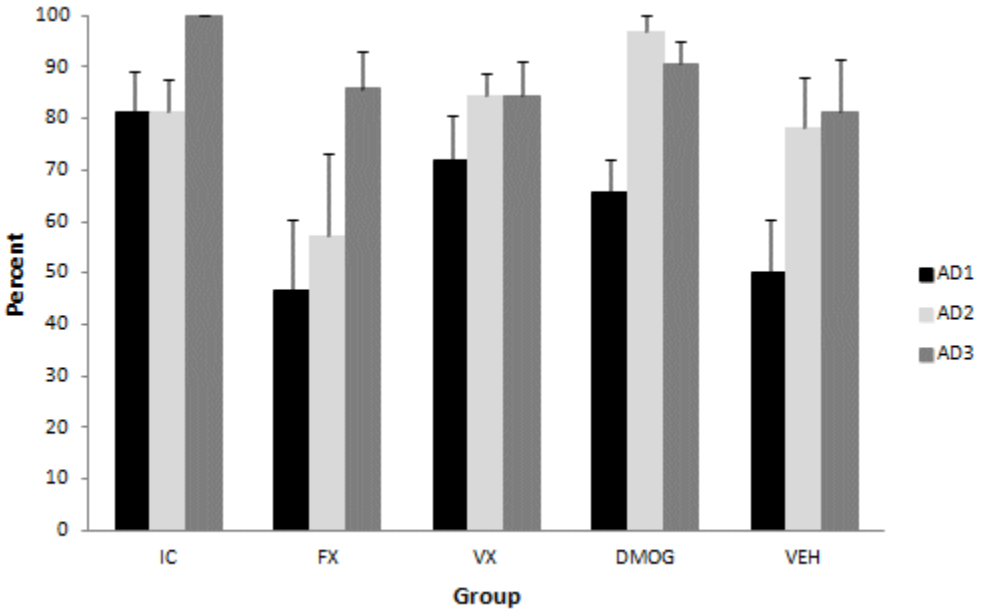


Figure 26. Acquisition phase – percent success by day. All animals increased the proportion of successful trials from acquisition day (AD) 1 to 3. However, no comparisons reached statistical significance.

Table 6.

Mean Percent Success (SEM) by Day During MWM Acquisition.

Group	Acquisition Day		
	1	2	3
IC	81.25 (7.83)	81.25 (6.25)	100.00 (0.00)
FX	46.43 (13.83)	57.14 (16.10)	85.71 (7.43)
VX	71.88 (8.76)	84.38 (4.57)	84.38 (6.58)
DMOG	65.63 (6.58)	96.88 (3.13)	90.63 (4.57)
VEH	50.00 (10.56)	78.13 (9.95)	81.25 (10.30)

Trials to criterion.

Trials to criterion is another measure to assess learning in the MWM. For this study, criterion was defined as two consecutive trials during which the difference in latency to find the platform was less than ten seconds (Kerr et al., 2010). As illustrated in Figure 27, VX (M = 5.25), IC (M = 5.375), and DMOG (M = 5..625) animals reached criterion in the fewest trials during the acquisition phase, whereas FX (M = 8.71) and VEH (M = 6.75) animals required more trials to consistently find the platform. A univariate ANOVA, however, showed no significant groups differences overall in trials to criterion ($F_{(4,34)}$ = 1.380, p = 0.262). Any effect of trials to criterion by group could be missed in the omnibus test because all animals did successfully acquire the task.

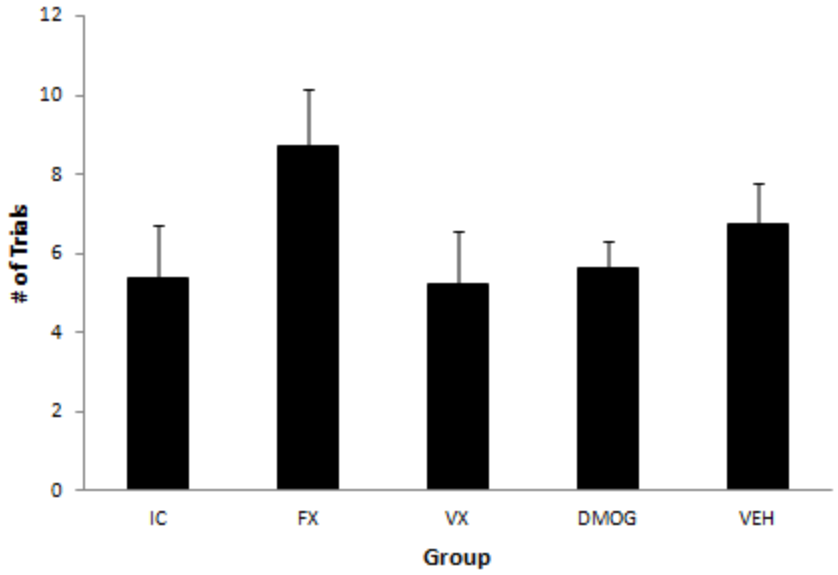


Figure 27. Acquisition Phase – Trials to Criterion. Although VX, DMOG, and IC animals took the fewest trials to reach criterion, there were no significant differences among groups in trials to criterion.

Swim distance and swim speed.

The results for percent change in latency, and the percent correct trials across the three acquisition days corroborate the swim distance data. All animals appeared to learn the task over the first three days as indicated by the increase in the number of successful trials. Therefore, swim distance across the three acquisition days would be expected to be similar among groups; swim distance should decrease across days because of increased success and decreases in latency to find the platform. This idea was supported by a repeated measures ANOVA with acquisition day as the repeated factor, which showed a significant effect of day ($F_{(2,33)} = 18.310$, $p < 0.001$), but no significant day by group interaction ($F_{(8,68)} = 0.970$, $p = 0.467$). Swim distance data by acquisition day is illustrated in Figure 28.

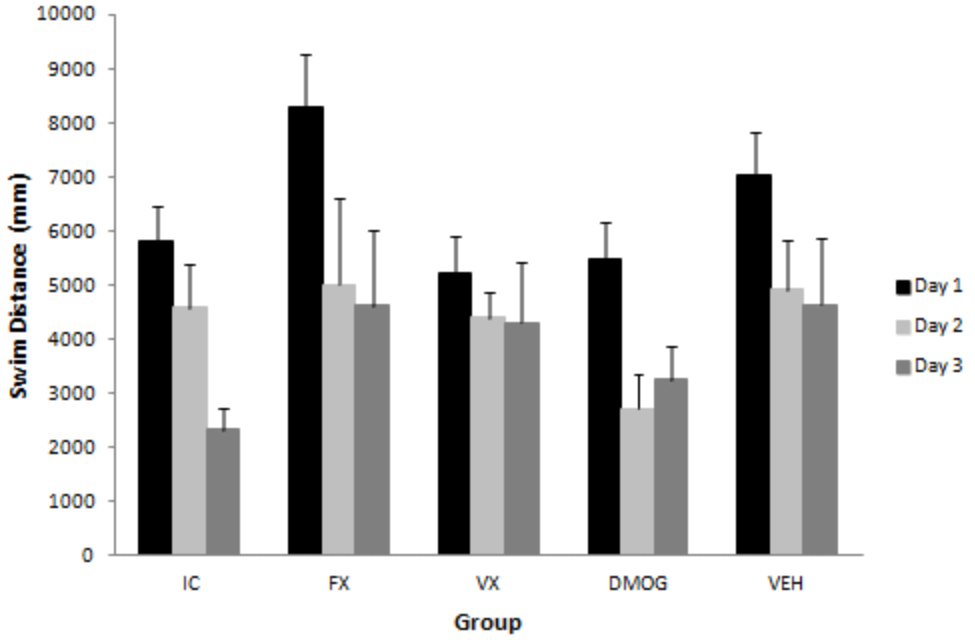


Figure 28. Swim distance by day during MWM acquisition. There were no significant differences in distance traveled while searching for the platform.

To counter the contention that exercising animals simply find the platform faster than non-exercising animals because of physical conditioning, a repeated measures ANOVA was performed on the swim speed data. Swim velocity data is illustrated in Figure 29. There was neither a significant main effect of day ($F_{(2,33)} = 0.652$, $p = 0.528$) nor a significant day by group interaction ($F_{(8,68)} = 1.171$, $p = 0.330$). Therefore, exposure to exercise before MWM training cannot explain any differences in MWM performance, particularly in latency to find the hidden platform.

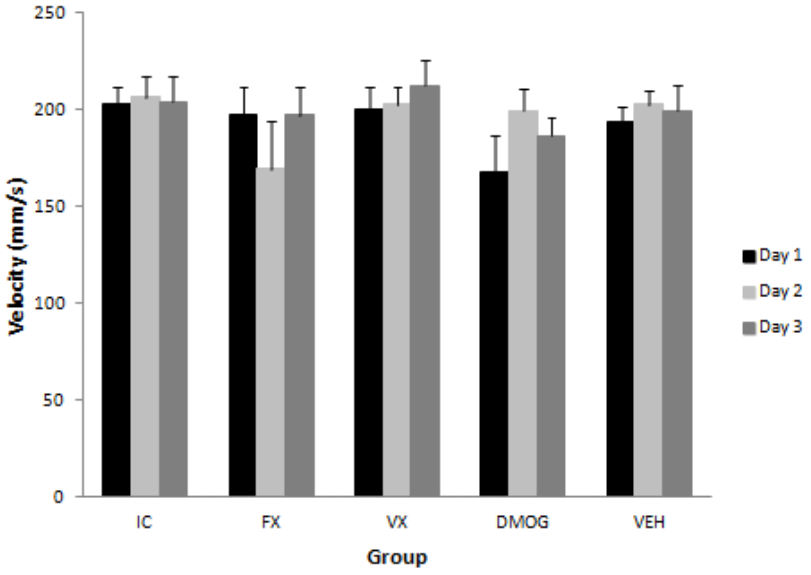


Figure 29. Swim velocity during MWM acquisition. No group differences in swim speed were detected.

Reacquisition phase.

The purpose of including a reacquisition phase during MWM training is to assess an animal's ability to assimilate it learning strategy to find the platform even after the location has changed. Small differences between latency to find the platform on days three (acquisition day three) and day four (reacquisition day 1) indicate that the animal has learned not only that there is a way to escape, but also that the animal has learned the strategy to find the escape platform.

Latency to find hidden platform.

Figure 30 shows the average latency to reach the platform on days four and five (reacquisition days 1 and 2) of MWM training. IC, VX, DMOG animals found the platform the fastest on the first day of reacquisition (M = 26.70, M = 21.66, and M = 26.38, respectively), but a repeated measures ANOVA with reacquisition day as the repeated factor did not show a significant difference among groups in latency to find the platform across the two days of reacquisition ($F_{(4.34)}$ = 1.314, p = 0.285). There was,

however, a significant effect of day ($F_{(1,34)} = 19.556$, $p < 0.001$) such that all animals improved their performance significantly from reacquisition day 1 to reacquisition day 2.

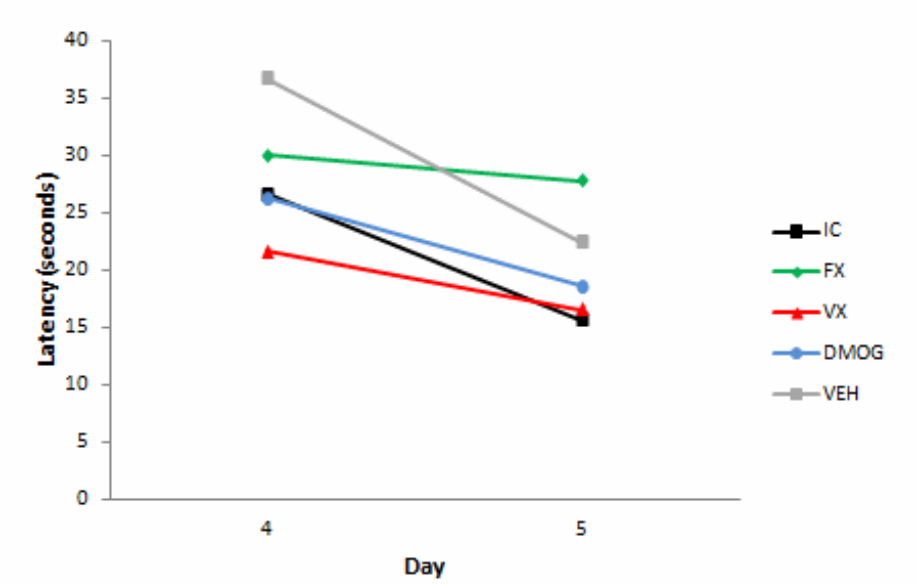


Figure 30. Reacquisition phase - latency to find the hidden platform. On day 4, FX and VEH were the slowest find the platform, but along with other groups, improved their performance on day 5. No statistical differences in overall reacquisition latency to find the platform were found.

If an animal learns within the first few trials of day four (reacquisition day 1), then we can conclude that the animal is demonstrating behavioral flexibility. Day four latency to find the platform by trial is presented in Figure 31. To investigate the differences in the animals' ability to assimilate their learning strategy to find the platform on the first day or reacquisition, a univarite ANOVA was used to compare average latency to find the platform on day four (see Figure 32). Although it appears that VX ($M = 21.66$) and IC ($M = 26.70$) animals perform better across the four trials on day four, there were no significant differences among groups in average latency to find the platform ($F_{(4,34)} = 1.695$, $p = 0.174$), and percent change in latency to find the platform from trial one to trial four on day four was also not significantly different among groups ($F_{(4,34)} = 1.897$, $p = 0.134$).

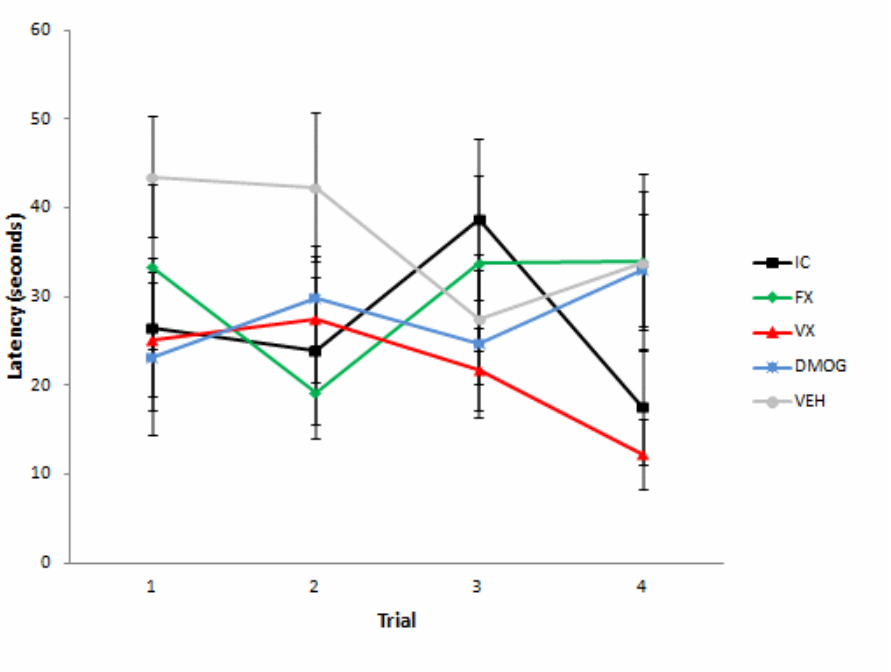


Figure 31. Reacquisition day 1 – Latency to find the hidden platform.

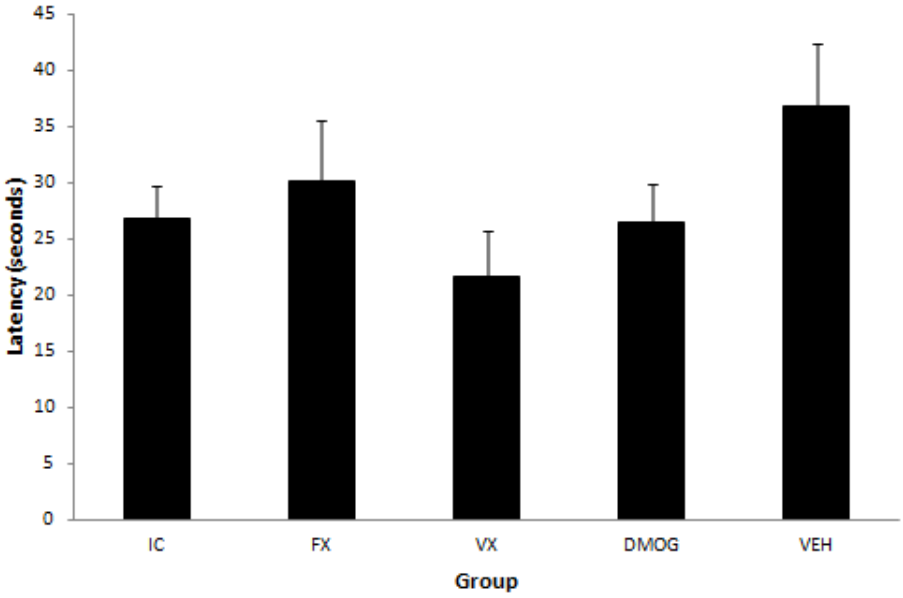


Figure 32. Average latency to find the platform on day 4 (reacquisition day 1). No significant group differences in average day 4 latency were found.

Percent success.

To further investigate reacquisition phase performance, a repeated measures ANOVA was conducted with reacquisition day as the repeated factor on the percent success data. This is an important analysis as latency may not always be the most appropriate measure of successful learning. The proportion of times the animals successfully finds the platform could be a more sensitive measure of proper acquisition. The omnibus test demonstrated that animals differed significantly in the percentage of successful trials during the reacquisition phase ($F_{(4,34)} = 3.227$, $p = 0.024$). As shown in Table 7 and Figure 33, the two exercising groups and the drug-treated groups had more successful trials during the reacquisition phase compared to IC and VEH animals. However, follow-up tests of between-subjects effects failed to reach statistical significance ($F_{(4, 34)} = 1.401$, $p = 0.255$). This failure to find a significant difference among groups could be because all animals improved their performance on day five (reacquisition day 2); this is particularly notable in the VEH group that went from just over chance performance, to approximately 90% correct on the second day of reacquisition.

Table 7.

Mean Percent Success (SEM) by Day During MWM Reacquisition.

	Reacquisition Day	
Group	1	2
IC	78.13 (5.67)	93.75 (4.09)
FX	82.14 (8.25)	78.57 (13.83)
VX	93.75 (4.09)	96.88 (3.13)
DMOG	81.25 (7.84)	90.63 (6.58)
VEH	56.25 (13.15)	90.63 (4.58)

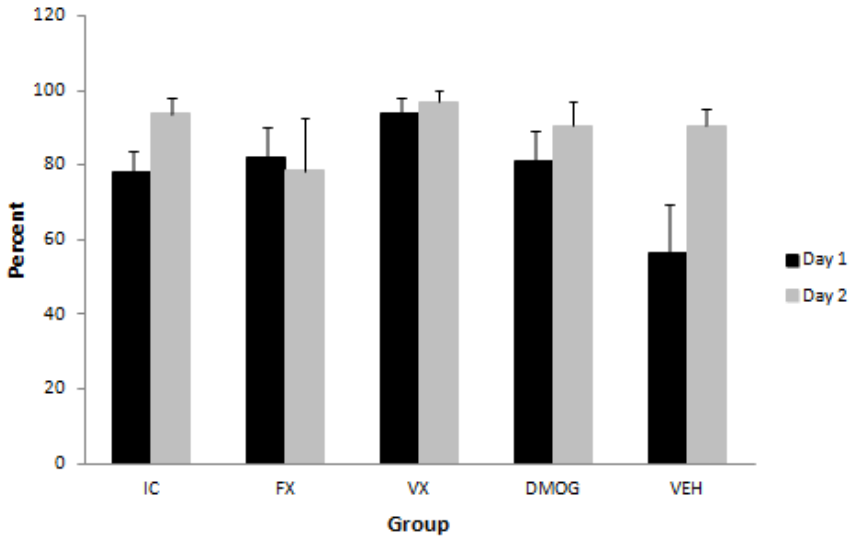


Figure 33. Reacquisition Percent Success. The omnibus test indicated significant differences among group (p = 0.024), but post-hoc tests failed to detect any significant differences between any groups.

Because the omnibus repeated measures statistics did reveal significant differences among groups in percent success during reacquisition, a finding that was likely due to performance on day four, a univariate ANOVA was performed to determine if there were significant differences in percent success on day four alone. This analysis showed a marginally significant effect of group on percent success during day four (F(4,34) = 2.476, p = 0.063). Multiple comparisons using the LSD correction showed that compared to VEH-treated animals, both exercising groups (VX, p = 0.004; FX, p = 0.049) and the DMOG-treated animals (p = 0.050), were significantly more successful in finding the hidden platform during reacquisition day one (see Figure 34).

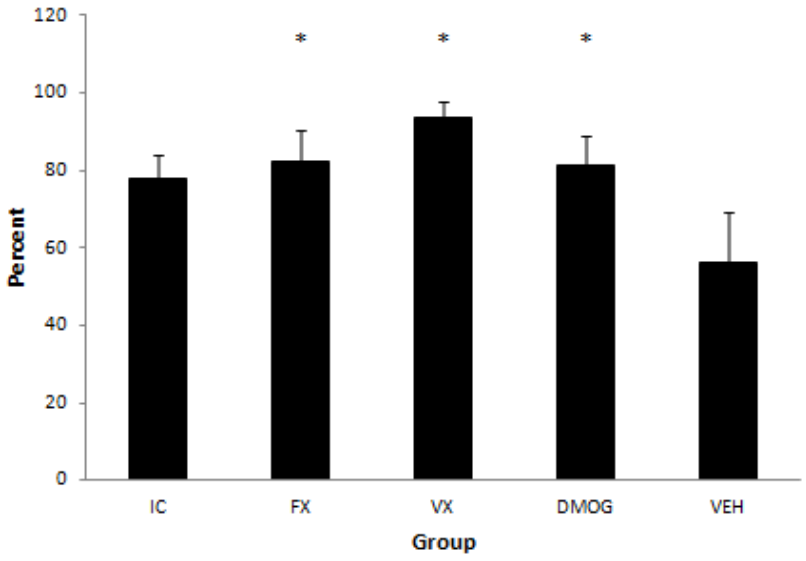


Figure 34. Percent success reacquisition day 1. A marginally significant effect of group on percent success was found, with exercising and DMOG animals significantly more successful compared to VEH animals ($p \leq 0.050$ for all comparisons).

Percent change latency to find the platform – reacquisition phase.

The percent change in average latency to find the hidden platform across the two reacquisition days was also investigated. FX animals changed their performance the least across the two days with just a 1.15% decrease in latency from day four to five. Similarly, VX animals had little change in latency to find the platform over the two reacquisition days (-9.36%). The other three groups of animals had larger decreases in latency between the two days: IC, -33.90%; DMOG, -29.96%; and VEH, -39.10%. These averages were analyzed using a repeated measures ANOVA with reacquisition day as the repeated factor, which showed no significant differences among groups ($F(4,34) = 1.207$, $p = 0.326$). This data is presented in Figure 35.

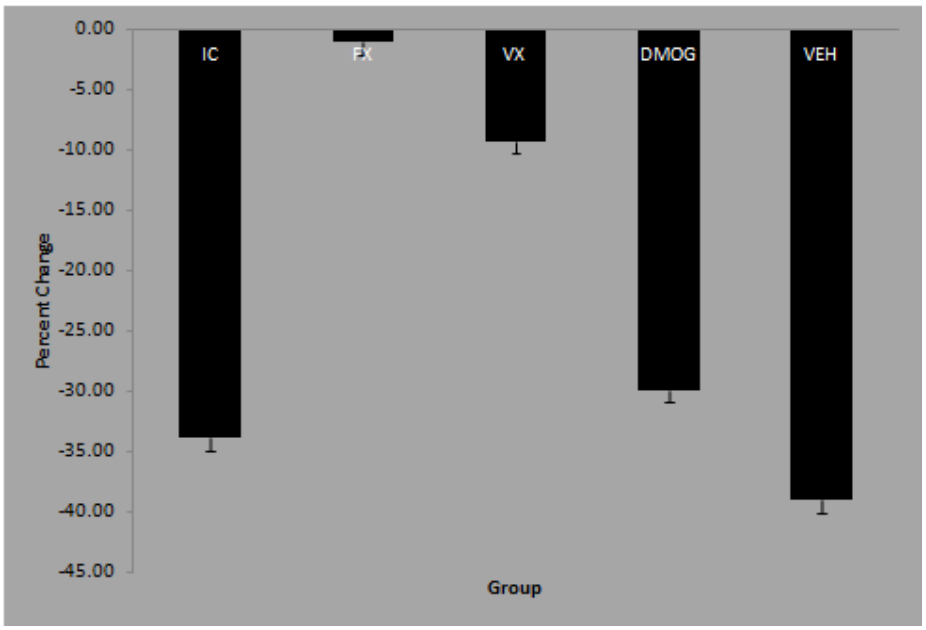


Figure 35. Percent change in latency to find the hidden platform during the reacquisition phase. No significant differences among groups were detected.

Percent change acquisition to reacquisition.

Another way to assess assimilation of learning strategy is to analyze the percent change in average latency to reach the platform between acquisition day three and reacquisition day one (see Figure 36). If an animal easily adapts to the change in platform location, a smaller percent change in latency will be recorded. VX animals demonstrated the smallest percent change in latency (34.78%), while all other groups of animals had much larger changes in times to find the hidden platform (IC = 121.20%, FX = 152.23%, DMOG = 123.83%, and VEH = 123.17%). A univariate ANOVA, however, showed no significant differences among groups in percent change in latency to find the platform from the last day of acquisition to the first day of reacquisition ($F_{(4,34)} = 0.290$, $p = 0.882$). The failure to find statistically significant differences in this case is likely due to the large variation in within groups, as illustrated in Figure 37.

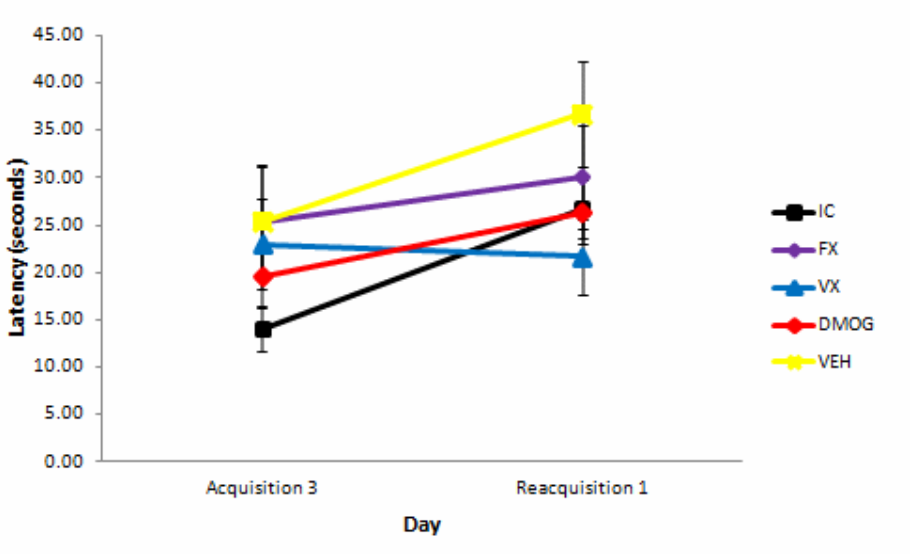


Figure 36. Latency – acquisition day 3 to reacquisition day 1.

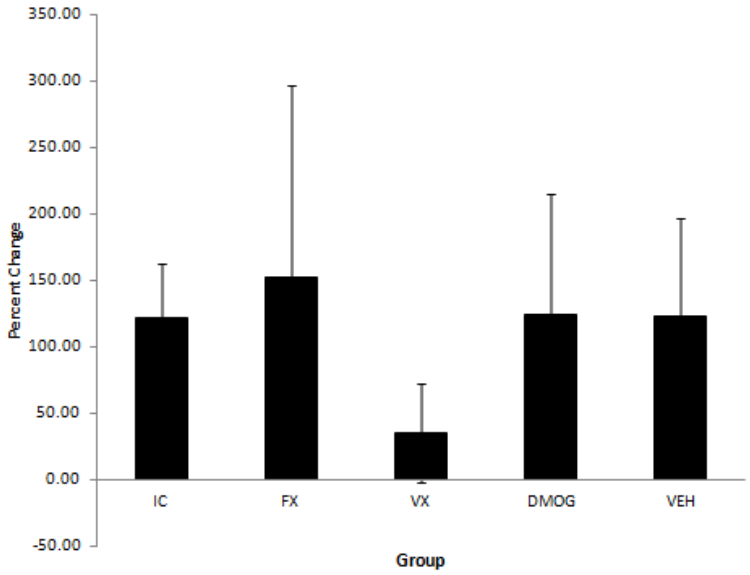


Figure 37. Percent change in latency to find the platform – acquisition day 3 to reacquisition day 1. There were no significant group differences in percent change in latency.

Again, due to the high degree of variability in the percent change acquisition to reacquisition data, we performed an outlier analysis in SPSS. Three extreme cases were detected – as defined by SPSS, those values more than three box-lengths from the edges of the box (Pallant, 2004) – one each from groups FX, DMOG, and VEH. After removal of these outliers,

there were still no significant differences among groups ($F_{(4,31)} = 1.557$, $p = 0.210$), but the data more closely adhere to the expected results (see *Figure 38*).

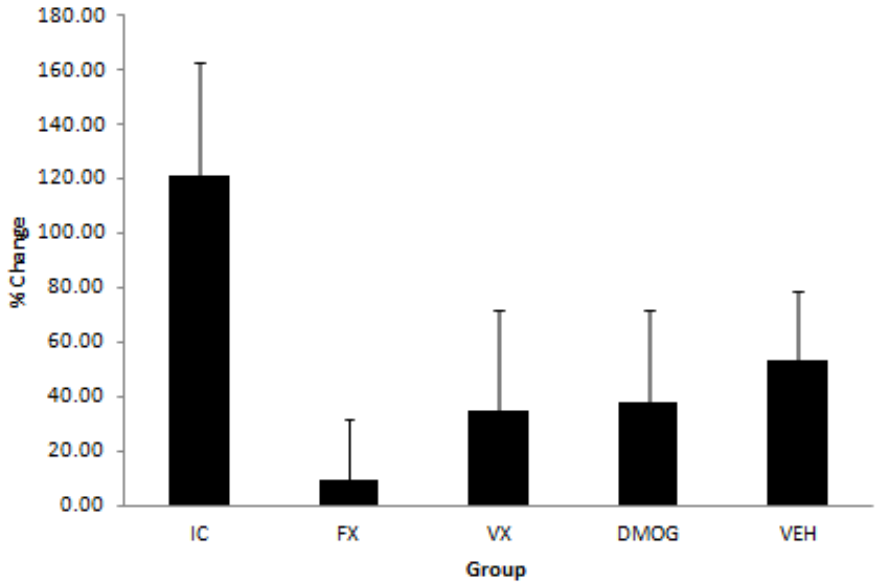


Figure 38. Percent change in latency to find the platform from acquisition day 3 to reacquisition day 1. Even after removal of outliers, there were still no significant differences in percent change in latency between the two phases of training.

Trials to criterion.

During the acquisition phase, FX animals took the longest to reach criterion, as defined by two consecutive trials in which the difference in latency to find the platform was less than 10 seconds. During reacquisition, however, VEH animals took the most trials to reach the defined criterion. Similar to the acquisition phase trials to criterion analysis, there were no significant differences among groups in trials to criterion during the reacquisition phase of training ($F_{(4,34)} = 1.342$, $p = 0.900$; see Figure 39).

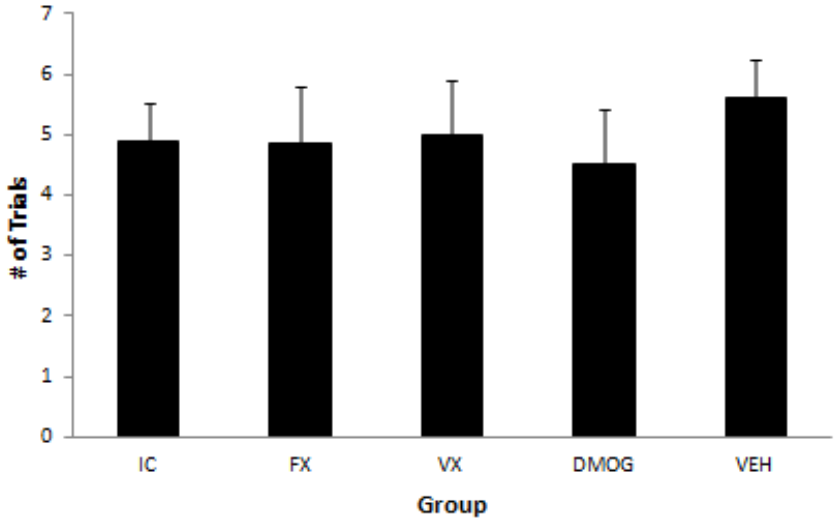
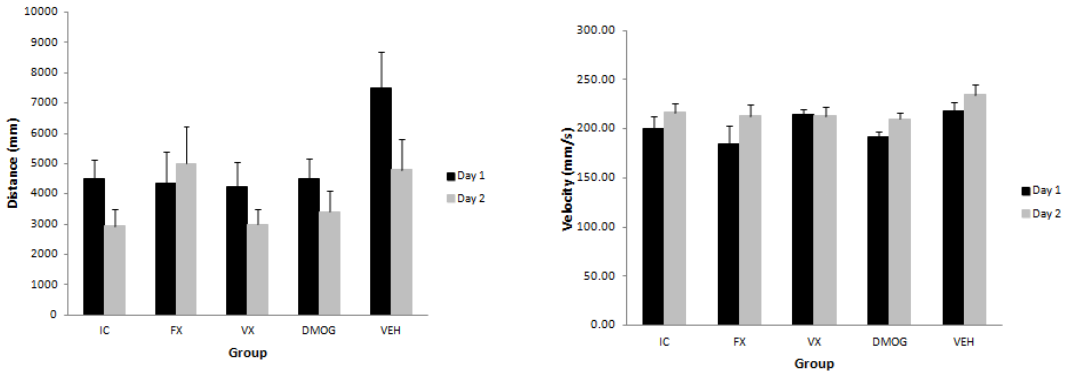


Figure 39. Reacquisition – Trials to Criterion. All animal groups attained criterion in a similar number of trials.

Swim distance and velocity.

Similarly to the acquisition phase of MWM training, there were no significant group differences in swim distance ($F_{(4,34)} = 2.082$, $p = 0.105$) or swim velocity ($F_{(4,34)} = 0.848$, $p = 0.505$). This data is shown in Figure 40.



A. B.

Figure 40. A) Swim distance by reacquisition day, B) swim speed by reacquisition day. There were no significant differences in swim distance or velocity.

MWM Retention.

Seven days after the final day of reacquisition training, animals underwent a single probe trial; the hidden platform had been removed from the pool prior to testing. During this trial, the animal was allowed to swim unhindered for 60 seconds. The purpose of this trial is to determine if the animal retained the previous location of the platform, as indicated by the amount of time the animal spends in the quadrant that previously contained the hidden platform during the reacquisition phase. Perseveration can also be examined by analyzing how much time the animal spends in the quadrant in which the platform was located during the acquisition, or initial, phase of training.

In the current study's design, the platform was located in the northeast (NE) quadrant during the acquisition phase of training (days 1-3). During the reacquisition phase on days four and five, the platform was moved to the opposite (southwest, SW) quadrant. Therefore, in the analyses described below, NE will be referred to as the acquisition quadrant, while SW will be referred to as the reacquisition quadrant.

All groups of animals spent between 20 and 30 percent of the 60 seconds exploring the reacquisition quadrant (IC = 22.99%; FX = 21.67%, VX = 29.16%; DMOG = 24.88%; VEH = 29.10%). As shown in Figure 41, although VX and VEH spent the most time in reacquisition quadrant, a univariate ANOVA showed that there were no significant differences among groups in percent time spent exploring the SW quadrant ($F_{(4,34)} = 1.581$, $p = 0.202$).

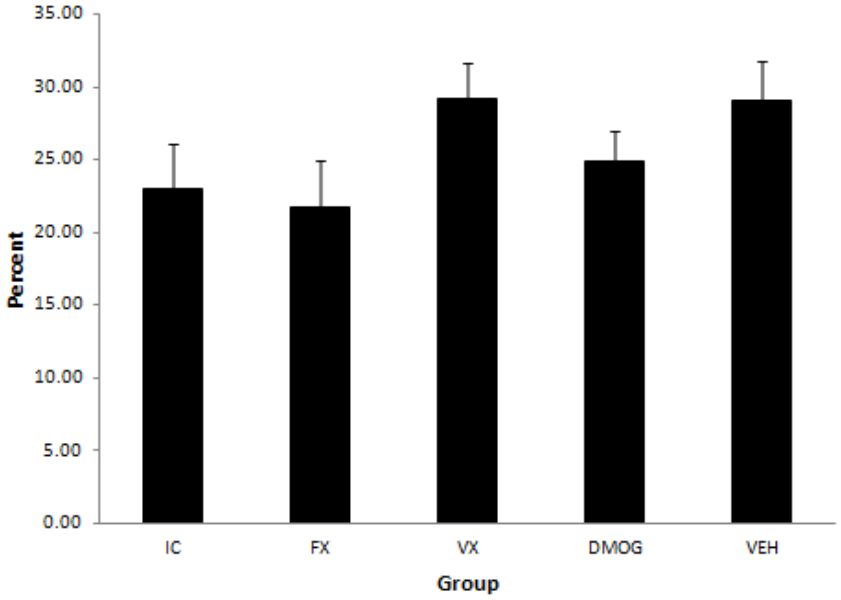


Figure 41. Percent time in previously correct quadrant (SW – reacquisition quad). There were no significant group differences in time spent in the reacquisition quadrant.

There was however, a significant difference among groups in time spent in the acquisition (NE) quadrant ($F_{(4,34)} = 2.728$, $p = 0.045$; see Figure 42). Post-hoc tests using the LSD correction showed that animals in both exercising groups, and the DMOG-treated animals spent significantly less time in the acquisition quadrant compared to VEH-treated animals (vs. FX, $p = 0.024$; vs. VX, $p = 0.005$; vs. DMOG, $p = 0.027$). IC animals also spent more time in the acquisition quadrant than exercising and DMOG animals, but none of these comparisons reached statistical significance; there was also no significant difference between IC and VEH animals in the amount of time spent in the acquisition quadrant ($p = 0.181$).

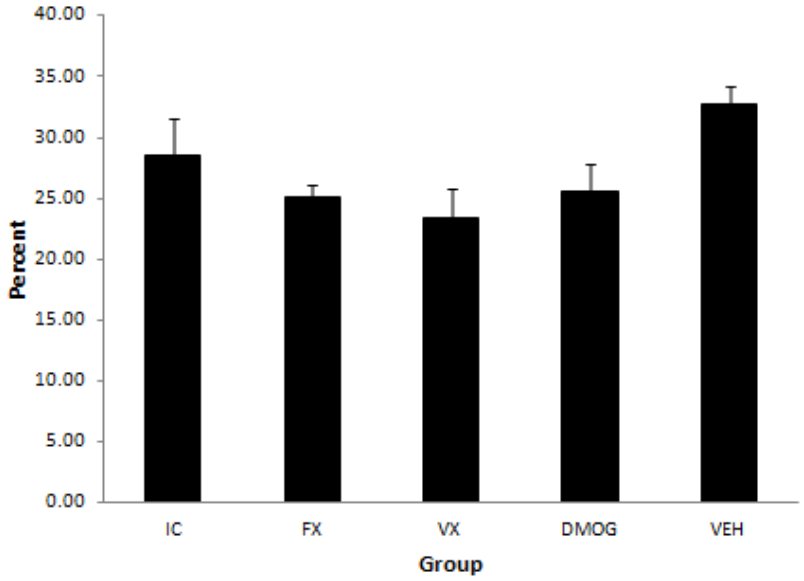


Figure 42. Percent time in acquisition quadrant. Both groups of exercising animals, and the drug-treated animals spent significantly less time in the initially reinforced quadrant. VEH and IC animals spent the most time in the acquisition quadrant.

Discussion

The purpose of these studies was to determine if exercise causes a transient period of hypoxia in the brain, specifically in the hippocampus, as assessed by the expression of the transcription factor HIF-1α. Furthermore, we sought to investigate whether expression of HIF-1α, or any downstream processes - specifically angiogenesis – would result in cognitive facilitation. In Experiment I, animals were exposed to either acute or chronic exercise and sacrificed at various time points following exercise to determine the timeline of exercise-induced hippocampal hypoxia. We found that HIF-1α is rapidly upregulated after an acute bout of exercise, suggesting that exercise causes a transient state of hypoxia in the brain.

Experiment II sought to compare and contrast the effects of exercise and the effects of direct manipulation of HIF-1α expression on cognitive performance. Of particular interest was whether HIF-1α expression itself and/or angiogenesis mediate

facilitation of learning and memory performance in the MWM in drug-treated and exercising animals. We found that voluntarily exercising, drug-treated, and surprisingly, inactive animals had the greatest blood vessel densities. Furthermore, these groups of animals performed the best in the MWM. This finding is consistent with our hypothesis that increases in hippocampal capillarity mediate cognitive facilitation in the MWM.

Experiment I.

Exercise-induced HIF-1α expression in the hippocampus.

Results from Experiment I showed that a single bout of moderately strenuous exercise causes a significant increase in the expression of HIF-1α protein in the hippocampus of the adult rat. HIF-1α is a transcription factor that is upregulated when normal oxygen tension decreases, and increases in HIF-1α expression result in the upregulation of various other factors, such as VEGF and EPO, which may in turn cause physiological changes that help return hypoxic tissue to normal oxygen homeostasis (Harten et al., 2010; Mu et al., 2003). Our results also show that the increase in HIF-1α protein occurs immediately after exercise cessation in area CA1, but not in areas CA2/3 and the DG. Furthermore, the effect appears to be biphasic in that protein expression increases immediately after exercise, then decreases at two hours post-exercise, and increases again at the 4-hour post-exercise time point. Finally, HIF-1α protein levels in the hippocampus are affected to a greater degree following acute exercise rather than chronic exercise, as demonstrated by our findings that protein expression in the long-term exercising group was lower than the 0- and 4-hour groups.

Our finding that area CA1 is the only region of the hippocampus that experiences exercise-induced increases in HIF-1α is consistent with findings reported in studies of

hypoxia and ischemia. Specifically, several studies have shown that following exposure to hypoxic conditions or stroke, a significantly greater degree of cell death, swelling, and excessive depolarization is observed in area CA1 compared to areas CA2/3 and DG (Bonnekoh et al., 1990; Kawasaki et al., 1990; Kriesman et al., 2000; Nitatori et al., 1995), suggesting that CA1 is more susceptible to decreases in oxygen compared to other hippocampal regions. Therefore, in an attempt to return hypoxic tissue to normal oxygen homeostasis, greater activation of HIF-1α and its target genes, such as VEGF and EPO, would be expected in this region. Our findings of exercise-induced increases in HIF-1α expression in CA1, but not CA2/3 or DG, are consistent with this reasoning.

There is conflicting data about the regulation of HIF-1α following exercise. Specifically, some evidence suggests that HIF-1α is regulated at the post-transcriptional level, such that exercise results in changes in protein expression, but not mRNA expression. Ameln et al. (2005) showed that immediately after knee-extension exercises, HIF-1α protein was significantly upregulated in muscle tissue, and remained elevated for six hours following exercise; however, these authors found no differences in HIF-1α mRNA at any post-exercise time point. Furthermore, another study failed to find significant changes in skeletal muscle mRNA at early time points following exercise, but did identify significant increases by 6 hours post-exercise (Lundby et al., 2006). In the brain, however, Kinni and colleagues (2011) identified increases in both protein and mRNA after chronic voluntary and forced exercise training. Our findings confirm that protein expression is altered immediately and four hours after exercise, indicating a biphasic effect. It could be that whatever transcripts were available are used up to affect a significant change in protein expression at the zero hour time point. This would deplete

the availability of transcripts, thereby causing a protein expression lag at the two-hour time point. By four hours, transcripts could again be available and result in significantly increased protein expression once again. This line of reasoning supports the notion that exercise-induced HIF-1α is regulated post-transcriptionally, and supports the findings of Ameln and colleagues (2005) who found significant increases in protein expression, but failed to find significant changes in mRNA expression. Future research with rodents should measure and compare the timelines of expression of HIF-1α protein and mRNA to more conclusively identify the level of HIF-1α regulation.

Our results are somewhat different than the findings of Kinni et al. (2011) who showed that three weeks of treadmill or voluntary exercise resulted in significant increases in both HIF-1α mRNA and protein in whole brain extracts. While we did not show significant increases in protein expression at the three week time point compared to the 0-, 2-, or 4-hours groups, our results do show significantly greater expression in the long-term animals compared to the 6-hour group. This difference in findings could be due to the different methodologies used. Specifically, it could be that the timeline of exercise-induced HIF-1α differs among brain structures, and since Kinni and colleagues used whole brain extracts to quantify protein and mRNA expression their signal could be from structures in which expression of HIF-1α may be delayed or prolonged relative to hippocampal expression. Previously, our lab has shown that different brain regions respond differently to exercise. Specifically, expression of the high-affinity VEGF receptor Flk-1 was found to be significantly upregulated in the cerebellum (paramedian lobule) just 2 days after the commencement of a voluntary exercise paradigm; Flk-1 expression in the motor cortex, however, did not increase significantly until day 10 of

exercise (Thompson et al., 2000). Furthermore, it is unclear when Kinni et al. (2011) extracted brain tissue after the last exercise session. We obtained brain tissue immediately after the last exercise session, which may be able to explain the small increase seen in the long-term animals' HIF-1α levels. It is certainly plausible that these differences in the timing of tissue collection could lead to the disagreement in the results of the present study and those of Kinni and colleagues.

Due to previous findings regarding HIF-1α expression in muscle tissue following chronic exercise, we did not expect to find a significant increase in HIF-1α levels in the chronically exercised animals. Specifically, in a study conducted by Lundby et al. (2006), human participants trained one leg in a knee extension exercise for three weeks. After this initial one-legged training, participants pedaled for three hours with both legs and HIF-1α mRNA was measured in both the previously trained leg and in the untrained leg. The authors found increases in mRNA in the untrained leg, but not the trained leg, six hours after exercise cessation. These data suggest that once the tissue becomes conditioned, increases in HIF-1α expression are no longer necessary to maintain normal oxygen homeostasis in the trained tissue such that increases would be observed in untrained tissue, but not in trained tissue. We propose this acclimation effect is due in part to angiogenesis, and the increased perfusion and the ultimate return to oxygen homeostasis in the affected tissue. Stated another way, activation of HIF-1α and the downstream genes (i.e., VEGF) which result in physiological changes at some point makes further increases in HIF-1α expression unnecessary and impossible due to adequate tissue oxygenation even during exercise.

The finding of increased HIF-1α expression in areas CA1 and CA2/3 in long-term inactive animals was surprising. The long-term animals were only approximately two weeks older than the short-term groups of animals, so age should not have been a contributing factor to these elevated levels. In fact, some evidence suggests that aged animals have decreased HIF-1α expression in response to hypoxic challenges (Ndubuizu et al., 2009, 2010), even after hypoxic pre-conditioning (Rabie et al., 2011) which has been shown to potentiate the HIF-1α response and therefore induce neuroprotection following subsequent changes in brain oxygenation (i.e., ischemia or hypoxia) in non-aged animals (Jones & Bergeron, 2001; Miller et al., 2001). Nevertheless, we are confident that the age difference between long- and short- term groups of animals was not great enough to result in differences in HIF-1α expression.

Some evidence does suggest that HIF-1α could be increased in response to environmental stress, such as exposure to hot or cold temperatures (reviewed in Kassahn, Crozier, Portner, & Caley, 2009), however, there is little evidence to suggest the possibility that the environment to which our animals were exposed would be sufficient to induce a stress-related HIF-1α response. The increase in HIF-1α in response to stress appears to involve a complex interaction among several pathways that are sensitive to the presence of reactive oxygen species (ROS). The increase in ROS results in alterations in the expressions of several molecules, such as heat shock proteins and immediate early genes, and the initiation of pathways sensitive to cellular redox states (reviewed in Kassahn et al., 2009). Based on the complexity of these pathways, and the seemingly non-aversive nature of simple treadmill exposure – inactive animals were simply placed on the treadmill apparatus and left undisturbed for 20 minutes for each exposure - it is

unlikely that the observed increase in HIF-1α in this group of animals is due to the stressful nature of the task. This line of reasoning is also supported by the finding that no other groups of inactive animals had significant increases in HIF-1α expression, which would have been expected if the exposure was stressful. Although the increase in HIF-1α expression observed in ICLT animals is anomalous, we are confident that this effect is not due to stress.

In recent decades, the effects of exercise training on the brain have been the focus of a great deal of research. For example, a great deal of evidence shows that participation in exercise leads to improvements in cognitive tasks (Erickson et al., 2011; Hillman et al., 2006; Kim et al., 2010; Vaynman, et al., 2004; Winter et al., 2007). Kim et al. (2010) showed that both young and aged exercising animals performed significantly better than young and aged control animals in a shock avoidance task and in the radial arm maze. Additionally, Vaynman and colleagues (2004) showed that exercising animals performed significantly better than sedentary animals in the Morris Water Maze (MWM). Furthermore, Kerr et al. (2010) showed that whereas blocking exercise-induced angiogenesis in animals resulted in significant deficits in acquisition performance in the MWM, blocking exercise-induced neurogenesis did not negatively affect MWM acquisition. These findings suggest that exercise results in cognitive facilitation, and that angiogenesis may be the process by which this facilitation occurs. Given that VEGF, a growth factor intimately involved in angiogenesis under normal (Ferrara et al., 1998; Ferrara, 2004; Fraser et al., 2000) and pathological conditions (Folkman, Watson, Ingber, & Hanahan, 1989; Plate, Breier, Weich, & Risau, 1992; Yancopoulos et al., 2000), contains a hypoxia response element and is a target of HIF-1α (Milosevic et al., 2007;

Sharp & Bernaudin, 2004), it is possible that HIF-1α activation and upregulation serve as upstream mediators of not only exercise-induced angiogenesis, but also exercise-induced cognitive facilitation.

In addition to exercise-induced cognitive enhancement in normal human and animal populations, there is also evidence that exercise can improve not only the neurological prognosis (Ding et al., 2004a; Griesbach et al., 2009; Matsuda et al., 2011), but also the functional recovery (Griesbach et al., 2004; Grealy et al., 1999; Ke et al., 2011; Kluding et al., 2011) of victims of brain injury or stroke. Ding and colleagues (2004a) showed significant decreases in infarct volumes in animals that exercised for 3 weeks prior to ischemia compared non-exercised control animals. These neurological and cognitive improvements could be due to increases in growth factors that have potentially neuroprotective properties. Matsuda et al. (2011) showed significant increases in midkine (MK), nerve growth factor (NGF), and platelet-endothelial cell adhesion molecule (PECAM-1), in animals that began exercising after undergoing ischemia. Griesbach et al. (2004) also showed significant increases in brain-derived neurotrophic factor (BDNF) in animals that began exercising 2 weeks after undergoing a fluid percussion injury. Given that exercise causes increases in neurotrophic factors in normal animals (Ding et al., 2006; Lou et al., 2008), and that we have shown that HIF-1α is also upregulated in response to exercise, it is possible that HIF-1α may be another molecule involved in improved behavioral and neurological outcomes following injury.

Conclusions.

We have shown that a single bout of treadmill exercise causes a significant increase in HIF-1α protein in the hippocampus of the adult rat, suggesting that exercise

causes a state of hypoxia in the brain. This effect is limited to area CA1 and is consistent with findings that CA1 is more susceptible to changes in oxygenation than CA2/3 or the DG. Exercise induces brain plasticity that is ultimately associated with greater cognitive functioning, and has also been shown to facilitate neuroprotection in cases of brain injury and stroke. It is important to study the mechanisms by which the brain adapts to altered endogenous and environmental conditions, and to develop a variety of methodologies to propagate research with such important clinical implications. Because HIF-1α is a common target of investigations regarding stroke and exposure to hypoxia, and because we have shown that HIF-1α is also upregulated following physical activity, exercise may provide a less-invasive and alternative method for the study of decreased brain oxygenation.

Experiment II

The purpose of Experiment II was to investigate whether manipulation of HIF-1α expression via a PHD inhibitor would result in angiogenesis and/or improve MWM performance. Increases in both HIF-1α expression and blood vessel density were found. Furthermore, manipulation of HIF-1α or exercise appear to improve MWM performance, but the relationship between exercise, HIF-1α, and angiogenesis is still not entirely clear based on the present data.

Anatomy.

HIF-1α expression.

The results of Experiment II show that animals in the VX, FX, and VEH conditions had the highest expression of HIF-1α in all areas of the hippocampus. In area CA1, exercising animals (both VX and FX) had significantly greater HIF-1α expression

compared to DMOG and IC animals. A similar pattern was found in area CA2/3, where FX animals had the highest levels of HIF-1α, followed by VX animals. Finally, in the DG, VEH animals had the highest HIF-1α expression, but VX and FX animals also had higher HIF-1α levels compared to IC animals.

These findings partially confirm the results of Experiment I where we showed significant increases in HIF-1α in exercising animals. Although Experiment I utilized a forced exercise paradigm only, and animals were sacrificed at varying time points shortly after exercise cessation, the results of Experiment II nonetheless confirm that exercise causes a state of hypoxia in the brain as indicated by increased HIF-1α levels.

Although there was no significant increase in HIF-1α in animals that had exercised for three weeks in Experiment I, animals that had exercised for one week in Experiment II did have significantly increased HIF-1α levels in area CA1 and in the DG compared to inactive control animals. This difference in findings between the two studies could be because three weeks, but not one week, of exercise is enough to result in a ceiling effect in that after the early stages of training, tissue is still hypoxic enough to require physiological modifications of blood flow and the required supporting structure – both of which may be regulated by alteration in HIF-1α expression - to return the tissue to normal oxygen levels. In this line of reasoning, HIF-α will continue to be expressed in greater levels until the necessary restructuring, or angiogenesis and erythropoiesis, is complete to such an extent that oxygen levels remain normal with the same level of exertion. This hypothesis is supported by studies investigating the effects of long-term hypoxic exposure and the expression of HIF-1α. For example, Chavez and colleagues (2000) housed animals in oxygen-deprivation conditions (10% oxygen) for varying times,

from 12 hours to 21 days. They found significant increases in HIF-1α expression beginning at 12 hours post-hypoxic exposure, and these increased mRNA levels remained elevated through day 14, but returned to baseline by day 21. Furthermore, they also measured VEGF mRNA and found similar increases beginning at 24 hours and also returning to baseline levels by day 21 of hypoxic exposure. These findings suggest that there is a time at which further increases in HIF-1α are either no longer possible, or no longer necessary to maintain normal functioning. As with the one week exercise paradigm used in the current study which appears to have caused significant hippocampal hypoxia, the environmental hypoxia induced in Chavez et al.'s (2000) study was not severe enough to permanently injure the animals, but severe enough to cause increases in HIF-1α, which presumably resulted in changes in downstream genes (such as VEGF and angiogenesis) and processes that are necessary to maintain health in oxygen-deprived conditions. In Experiment II, these processes can be identified by the increases in blood vessel densities in the voluntary exercise animals who also displayed greater levels of HIF-1α compared to inactive control animals.

Surprisingly, the HIF-1α expression of DMOG-treated animals was not significantly increased compared to vehicle treated animals in any region of the hippocampus, and levels of HIF-1 in drug-treated animals was greater only compared to inactive animals (but this difference was not significant). The DMOG dosage for the current study was obtained from research conducted by Ogle and colleagues (2012) who found that a single intraperitoneal injection of DMOG resulted in significantly increased HIF-1α expression 12 and 24 hours after the injection. In order the match the experimental period of the exercising animals who exercised for one week, the agonist

was injected one time per day for one week. Because we used a prolonged drug-injection protocol, the difference in duration of injections could explain why we did not observe significant increases in HIF-1α compared to vehicle-treated and inactive control animals. We speculate that a negative feedback loop may have interfered with further increase in HIF-1α expression after a few days of treatment with the agonist. This hypothesis might also explain why chronically exercised animals did not have significant increases in HIF-1α expression in Experiment I, and why HIF-1α and VEGF mRNA expression decreases to baseline levels after prolonged exposure to hypoxia (Chavez et al., 2000). Another similar possibility for failure to detect increases in HIF-1α expression is that midway through the one week drug treatment period, it is possible that HIF-1α had reached maximum expression levels, and what we are observing is an increase over a newly established baseline. If this is the case, the increase over the new baseline may have occurred late enough in the treatment period such that there was insufficient time to build up to the observed expression levels in exercising animals. A future study could be done to investigate whether a single dose of DMOG, as described by Ogle et al. (2012), would cause significant increases in HIF-1α, and if this increase in expression is comparable to the exercise-induced increases that we observed in Experiment II.

Another unexpected finding from Experiment II was the level of HIF-1α expression in the vehicle-treated animals. As described above, the HIF-1α expression in this group of animals was similar to those of VX and FX animals. It could be argued that vehicle injections were stressful, and therefore may have caused increases in HIF-1α. If this was the case however, we would have expected to see increased expression in DMOG animals as well. As described above, there is little evidence that stress would

cause significant changes in HIF-1α expression in the brain and that if HIF-1α is activated in response to non-hypoxic environmental stressors, it requires the interplay of several genes (Kassahn et al., 2009). Administration of injections occurred very quickly, so we are confident that the momentary discomfort caused during the injections is not enough to induce a major stress response, or one that was sufficient to induce the observed large increases in HIF-1α expression. Despite the failure to detect increased HIF-1α levels in drug-treated animals, exercise and drug treatment, but not vehicle treatment, clearly had an effect on hippocampal blood vessel density.

Blood vessel density.

Our findings regarding blood vessel density are somewhat clearer than the HIF-1α data. We showed that animals treated with the HIF-1α agonist, voluntarily exercising animals, and IC animals had the greatest capillary densities in CA1 and the DG. There were no differences in blood vessel density among groups in area CA2/3. It is also important to note that FX and vehicle-treated animals had the lowest blood vessel densities in all three hippocampal regions studied.

Angiogenesis occurs in the hippocampus in response to both short and long-term voluntary exercise. Van der Borght et al. (2009) found significant angiogenesis in the hippocampus in animals that had exercised for just three days. Furthermore, Ekstrand and colleagues (2008) demonstrated that chronic exercise (three weeks) caused significant increases in newly born endothelial cells in rats. Our findings of increased blood vessel densities in the hippocampus in voluntarily exercising animals replicate the findings of these studies.

The finding of significantly decreased blood vessel density in animals forced to exercise was interesting, but not entirely unexpected. There is some evidence that treadmill exercise is stressful for rats (Contartaze, Manchado, Gobatto, & de Mello, 2008; Moraska, Deak, Spencer, Roth, & Fleshner, 2000). Increased levels of corticosteroids and changes in glandular weights are common signs of chronic stress (Koko, Djordjeviae, Cvijiae, & Davidoviae, 2004; Marin, Cruz, & Palenta, 2007; Soldani et al., 1999) and have been identified in animals trained in forced exercise paradigms. Specifically, Contartaze et al. (2008) reported that rats submitted to forced swimming exercise and forced treadmill exercise had significantly higher corticosterone levels in serum compared to control animals. Another study found significant decreases in the weight of the thymus gland and significantly increased adrenal gland weight in animals that underwent eight weeks of high-intensity treadmill exercise (Moraska et al., 2000). Aside from the peripheral effects of stress, there is some evidence that overly stressful training paradigms can abate cellular and structural changes in the brain as well. For example, Lou et al. (2008) exposed animals to low, moderate, or high-intensity treadmill exercise and found that only animals exercising at low intensity (maximum speed: 11 m/minute) had significantly greater BrdU-positive cells in the dentate gyrus compared to sedentary control animals. Additionally, moderate (maximum speed 14 m/minute) and high-intensity (maximum speed: 22 m/minute) exercisers had significantly fewer BrdU/NeuN double-labeled cells compared to low intensity exercisers. Finally, and of particular interest to the current study, Lou and colleagues reported significantly increased Flk-1 mRNA expression in the low-intensity group compared to controls, but not in the moderate or high-intensity groups. These results suggest that increasing the intensity of a

workout may assuage the useful physiological and cellular changes elicited by exercise. The FX animals in Experiment II of the current study were run at a speed (15 m/minute) in between the moderate and high-intensity groups in Lou et al's study. Therefore, it is reasonable to conclude that the lower blood vessel densities found in the FX group are due to the somewhat stressful nature of the treadmill exercise paradigm and a possible decrease in Flk-1 expression as described by Lou et al. (2008). An interesting follow-up experiment would be to replicate the study of Lou et al. and vary the intensity of exercise, and measure other downstream targets of HIF-1α such as VEGF and its two high affinity receptors, Flk-1 and Flt-1, to determine the molecular mechanisms underlying exercise-induced structural changes following varying degrees of effort. Because we did find increased levels of HIF-1α and increased blood vessel density in voluntarily exercising animals, it doesn't seem likely that exercise must be particularly intense to affect changes in HIF-1α expression or angiogenesis. Therefore, a less stressful treadmill exercise paradigm may result in similar changes in capillarity as those observed in VX animals.

Because VEGF contains a hypoxia response element and is a target of HIF-1α (Milosevic et al., 2007; Sharp & Bernaudin, 2004), and because VEGF is intimately involved in angiogenesis (Ferrara, 2004; Risau, 1997), we hypothesized that administration of a drug agonist of HIF-1α would cause angiogenesis. Our results support this hypothesis; animals treated with DMOG had significantly greater blood vessel density compared to FX animals in areas CA1 and the DG. Drug-treated animals also had higher blood vessel density compared to VEH-treated animals in each region of hippocampus, but these differences did not reach statistical significance. After qualitative examination of the data, and when taking into account the large standard error

measures in the VEH group, we are confident that with more animals, and therefore more statistical power, the differences in capillary density between drug- and vehicle-treated animals would reach statistical significance.

Just as the HIF-1α expression of VEH animals was much higher than we hypothesized, we also found high blood vessel densities in the inactive control animals. This increase in capillarity is surprising, but it is worth mention that standard error measures of blood vessel density in the IC group were the largest among all of the groups in all hippocampal regions analyzed. The variability within this group of animals may partially explain the inflated density measurement. However, it is important to note that the behavioral performance of the IC animals was also surprisingly good.

MWM.

The MWM paradigm we used included three phases: acquisition, reacquisition, and retention. We chose this particular design as it allows us to explore changes in different types of learning and memory. Along with assessing an animal's ability to efficiently acquire the task, reacquisition allows for an analysis of behavioral flexibility – specifically of interest is whether or not animals learn the strategy to find the hidden platform, or simply remember where the platform is located without having learned the rule about how to find it. This situation would be apparent if animals continue to look for the platform in a previously reinforced quadrant even after the platform had been moved to a different location within the maze. Furthermore, a remote probe trial tests the animal's ability to retain information about how to successfully perform the task.

The MWM can be conducted in several different ways, all of which provide varying levels of difficulty. In recent research, the majority of studies have utilized a two

trials per day design (Gomez-Pinilla, et al., 2008; Griesbach et al., 2009; Kerr et al., 2010; van Praag et al., 1999; Vaynman et al., 2004), whereas fewer studies, including in the present study, used a four trials per day paradigm (Haege et al., 2010; Khorshidahmad et al., 2012; Mu, Li, Yao, & Zhou, 1999). Task difficulty is often thought to depend on the number of trials per day. To ensure that animals would acquire the task, we chose to use four trials per day, but our data indicate that perhaps a more difficult two trial per day paradigm would have been more sensitive to treatment-induced changes in cognitive performance.

Acquisition.

A qualitative review of the acquisition phase data indicate that VX, DMOG, and IC animals were the most efficient in finding the hidden platform, whereas FX and VEH animals took the longest to find the platform. Although the repeated measures ANOVA failed to detect significant differences among groups across all three days of acquisition training, there was a significant difference in average latency to find the platform on day one. Specifically, FX animals were significantly slower to find the platform compared to VX, DMOG, and IC animals. Furthermore, there was a marginally significant difference among groups in the proportion of successful trials on day 1; IC and VX animals were successful more often than FX animals. These differences in performance on the first day of training suggest that inactive, voluntarily exercising, and drug-treated animals acquired the task more quickly than animals forced to exercise.

Other analyses of acquisition data also failed to detect significant group differences. There was no difference in percent change in latency to find the platform throughout the acquisition phase, and there were also no differences among groups in

trials to reach criterion. For the purposes of this study, we defined trials to criterion in the same way as described by Kerr et al. (2010), where criterion performance was defined as two consecutive trials during which the difference in latency to find the platform was less than 10 seconds. Although no significant differences were found on this measure, the trials to criterion data follow the same pattern as the latency data with VX, DMOG, and IC animals reaching criterion in fewer trials than FX and VEH animals. There were also no differences in swim distance or swim speed, which indicates that any differences that would be observed in latency to find the platform are not due to differences in swimming ability; this finding counters the argument that exercising animals are simply more physically fit and therefore able to reach the platform faster compared to non-exercising animals.

The failure of the repeated measure statistic to detect additional significant differences across the acquisition phase of training could be because all groups of animals did acquire the task as indicated by decreased latencies on each subsequent acquisition day, and the significant main effect of day during this phase of training. Another factor in these null findings could be that the task was not difficult enough. If all groups of animals acquired the task during the three days of acquisition, then it could be that a four trial per day MWM paradigm is not sensitive enough to detect any significant treatment-induced differences in acquisition performance. In fact, most studies use a more challenging two trial per day design, and all of these studies have found significant group differences between exercising and sedentary animals (Gomez-Pinilla, et al., 2008; Griesbach et al., 2009; Kerr et al., 2010; van Praag et al., 1999; Vaynman et al., 2004). Based on these previous findings, and given the apparent qualitative differences in

acquisition performance and the statistically significant difference in average latency on day one, it is likely that had we used a more challenging MWM protocol, group differences in more of the measures of interest would have reached statistical significance.

In considering all of the data from the acquisition phase of training, the only measure to reach statistical significance between groups was the average latency to find the platform on day one. Furthermore, the FX animals were the slowest to find the platform on this initial day of training. This deficit in task acquisition could be attributable to the stressful nature of treadmill exercise as described above.

Reacquisition.

As described previously, the purpose of the reacquisition phase of MWM training is to determine whether or not the animal has learned the strategy to escape the pool. If an animal uses the environmental cues to navigate to find the hidden platform, then it should be able to quickly find the platform after just a few trials, even after the platform changes location. This type of performance would suggest behavioral flexibility in that during the first trial on day four, the animal will have learned that the platform had been moved, but would still be able to find it quickly on subsequent trials through the use of the environmental cues. Poor performance on the first day of reacquisition suggests that the animal has simply remembered the previous location of the platform without fully acquiring the strategy to find it by using the cues. This type of behavior would be considered perseverative, and shows a failure of the animal to alter its behavior despite the new demands of the task.

Similar to performance during the acquisition phase of MWM training, during the reacquisition phase, inactive, voluntarily exercising, and drug-treated animals were the fastest to find the hidden platform on day four (reacquisition day 1) of training. However, statistical tests again failed to detect significant differences in latency to find the platform across both reacquisition days, and also did not show significant differences in day four average latency. Other relevant measures such as percent time in the previously correct quadrant, percent time in the correct quadrant, previously correct quadrant entries, correct quadrant entries, trials to criterion, and swim distance and velocity, were also not significantly different among groups.

Although all groups took similar amounts of time to find the hidden platform during this phase, there were marginally significant differences in the proportion of times the animals successfully found the hidden platform. Specifically, the omnibus F-statistic did indicate significant differences among groups in percent success over both reacquisition days, but post-hoc tests to determine specific between-groups differences failed to reach significance. However, if considering only day four, VX, FX, and DMOG trended toward greater success in finding the platform compared to vehicle-treated animals. The failure to find outright significant differences in the percent success measure across both days of reacquisition is likely due to the fact that all groups improved their performance on the second day of reacquisition; with the exception of the FX group in which average percent success reached approximately 80%, all other groups were on average successful in finding the platform over 90% of trials on day five (reacquisition day 2). This improvement likely resulted in failure to detect specific between-groups differences in post-hoc tests even when the omnibus test indicated

significant group differences. The large degree of variability in the VEH group on day four could also explain the trend toward the greater success of VX, FX, and DMOG animals on day four alone. Based on the reported pattern of data, it is likely that larger groups of animals and therefore greater statistical power would yield statistically significant group differences. Furthermore, a more difficult MWM paradigm may have been more sensitive in detecting these group differences.

Based on data from the acquisition phase of the MWM, the finding that VX and DMOG-treated animals had greater percent success in the reacquisition phase than VEH control animals was not unexpected. However, given their poor performance during acquisition, we did not expect the FX animals to have greater percent success than any other group. It appears that FX animals are somewhat retarded in their initial acquisition of the task, but after acquisition day one, they perform similarly to all other groups of animals, and their performance during reacquisition surpasses that of vehicle control animals, as indicated not only by increased percent success, but also faster latency to find the platform during reacquisition. Additionally, compared to IC animals, FX animals have much less difference between performance on acquisition day three and reacquisition day one. These observations regarding the pattern of behavior by FX animals suggests that while forced exercise may decrease the angiogenic response, and result in impairments during initial phases of learning, forced exercise may still facilitate performance in the long run, particularly in terms of behavioral flexibility. The large degree of difference between day one and day three latencies to find the platform among FX animals indicates that after that initial learning, they are able to match the performances of other groups of animals that did not experience the initial learning

impairment. Furthermore, they do not seem to show significant impairment, or perseveration, during the reacquisition phase as their percent change in latency to reach the platform from day three to day four (reacquisition day one) did not change to the extent that VEH and IC animals did. Of course, these conclusions should be considered cautiously as the differences described here did not reach statistical significance.

The large percent change in latency to find the platform between acquisition day three and reacquisition day one in VEH and IC animals suggests behavioral perseveration. Although IC animals were among animals to learn the task the fastest, this group of animals was somewhat impaired on reacquisition day one compared to exercising and drug-treated animals. These results suggest that angiogenesis alone, even in the absence of exercise, may be most important during initial task acquisition, but is not as important in behavioral flexibility. This hypothesis is supported by the findings that despite their low blood vessel densities, FX animals still displayed behavioral flexibility as indicated by smaller changes in performance between day three and day four of training. It is possible that other exercise-induced brain processes play a larger role than angiogenesis in behavioral flexibility specifically.

Retention.

Seven days after the final reacquisition training day, animals underwent a single probe trial. The purpose of this trial was to determine if the animal retained the memory for where the platform was located on the final day of training. Measures of interest for this test include percent time spent in the reacquisition quadrant and percent time spent in the acquisition quadrant.

Results from the retention probe showed no significant differences in the percent time spent in the previously correct quadrant. All groups of animals spent between 20 and 30 percent of the 60 seconds in the quadrant in which the platform had been located during reacquisition training. This finding indicates that treatment did not affect animals' trace memory for the platform's previous location. On the other hand, there was a significant difference among groups in the percent time spent in the originally trained quadrant; VEH and IC animals spent the most time in this quadrant. This behavior could be interpreted as perseveration. Even after reacquisition training, these animals continue to spend the most time in a quadrant that had not been reinforced on the most recent MWM exposure.

In addition to spending the most time in the non-previously-reinforced quadrant during the retention test, IC and VEH animals also had the largest increases in latency from acquisition day three to reacquisition day one. These findings, when considered together, indicate behavioral inflexibility, or perseveration. On the other hand, the exercising and DMOG animals do not appear to perseverate, as indicated by smaller differences in latency to the platform between the two phases of training, and also by significantly less time spent in the originally reinforced quadrant.

Anatomy and behavioral performance.

The purpose of investigating the behavioral performance of exercising and drug-treated animals in this experiment was to determine if the physiological changes brought about by exercise would result in significant differences in behavioral performance, which has been previously demonstrated (Gomez-Pinilla, et al., 2008; Griesbach et al., 2009; Kerr et al., 2010; van Praag et al., 1999; Vaynman et al., 2004). We also

investigated the role of HIF-1α expression in cognitive facilitation. In an attempt to support the findings of Kerr et al. (2010) who showed that inhibition of angiogenesis disrupted normal learning performance in the MWM, we sought to determine if manipulation of HIF-1α expression would alter behavioral performance, perhaps via increases in capillarity in the hippocampus.

Our results show that voluntarily exercising animals, DMOG-treated animals, and inactive control animals had the highest blood vessel densities in the hippocampus. Furthermore, the MWM results show that during acquisition the VX, DMOG, and IC groups performed the best. Notably, FX animals had the lowest blood vessel densities and were the slowest on average to reach the platform on day one of training. Taken together, these findings indicate that blood vessel density may mediate acquisition performance in the MWM. This hypothesis corroborates the findings of Kerr et al. (2010) regarding the role of angiogenesis in exercise-induced cognitive facilitation.

We also found that despite slower learning during the acquisition phase of training, the FX animals matched the performance of other groups by the end of acquisition. Furthermore, FX animals along with the VX and DMOG groups were more successful in finding the hidden platform on day one of reacquisition. Finally, both exercising groups and the DMOG animals spent the least amount of time in the acquisition quadrant during the retention probe test. These results suggest that while decreased blood vessel density may impair acquisition, it does not appear to affect behavioral flexibility or retention.

Interestingly, we found that exercising animals and vehicle-treated animals had the highest levels of HIF-1α expression, and DMOG-treated and IC animals had the

lowest HIF-1 levels. VEH animals, with their high levels of hippocampal HIF-1α, performed poorly in the MWM, whereas DMOG animals, with low HIF-1α expression, performed very well in the MWM. These data indicate that although exercise does increase HIF-1α expression as demonstrated in both Experiment I and Experiment II, expression of the transcription factor itself does not appear to play a role in cognitive facilitation. Stated another way, because we did not observe an increase in HIF-1α expression in drug-treated animals, we cannot conclude that HIF-1α itself is a mediator of exercise-induced cognitive facilitation. However, treatment with DMOG did result in increased blood vessel density, which suggests that although HIF-1α cannot be directly implicated in behavioral performance, downstream changes to vasculature caused by manipulation of the HIF-1α pathway do appear to be involved in improved MWM acquisition.

Our findings are somewhat similar to the findings of Adamcio et al. (2010) who showed that treatment with the PHD inhibitor FG-4497 resulted in significant improvements in contextual fear conditioning three and four weeks after drug treatment. However, whereas these authors did not find significant behavioral differences one week after drug treatment, the improvements that we observed in the MWM occurred during training beginning just 24 hours after the final drug treatment. The lag in time between drug treatment and cognitive enhancement reported by Adamcio et al. (2010) is puzzling. It is possible that the pharmacokinetic and pharmacodynamic properties of the two drugs differ significantly. For example, one drug may act more quickly, or may be metabolized or degrade more slowly when compared to the other drug. Our findings of increased angiogenesis and improved cognitive performance in DMOG animals compared to

vehicle-treated animals considered together with the delayed cognitive facilitation shown by Adamcio et al., suggest that DMOG produces downstream physiological effect more quickly than FG-4497.

Conclusions.

In Experiment II, we have demonstrated that both forced and voluntary exercise result in significant increases in HIF-1α expression. These findings replicate the findings of Experiment I, and further the timeline for the exercise-induced increase in HIF-1α expression. Based on the findings of Experiment I where levels of HIF-1α returned to baseline in the group of animals sacrificed at six hours post-exercise, it was unclear whether or not there would be significant increases in HIF-1α expression after a whole week of exercise. Chavez et al. (2000) found that by day 21 of housing in hypoxic conditions, HIF-1α mRNA levels had returned to baseline. Lundby and colleagues (2006) also found that chronic exercise training did not increase muscular HIF-1α expression in human subjects. Since we did not observe an increase in HIF-1α labeling in the long-term exercise group in Experiment I, the results from Experiment II that one week of either forced or voluntary exercise caused increases HIF-1α labeling was interesting. It is possible that even after one week of exercise training, tissue oxygenation is still compromised enough to cause increases in hypoxia-inducible molecules. At later time points, however, we hypothesize that the downstream effects of HIF-1α and its target genes would have affected significant changes in blood flow and vasculature such that exercise no longer causes hypoxia, and therefore no further increases in HIF-1α would be observed.

Behavioral testing in Experiment II showed that blood vessel density appears to be an important mediator of acquisition performance; groups with the highest capillarity performed the best during the acquisition phase. These findings corroborate the findings of Kerr et al. (2010) who showed that inhibition of angiogenesis resulted in severe deficits during acquisition in the MWM. Furthermore, we have shown that while blood vessel density is important for acquisition, it does not appear to significantly affect behavioral flexibility. Both exercising groups of animals and the drug-treated animals were more successful in finding the platform on the first day of reacquisition training. Additionally, these three groups of animals spent the least amount of time in the acquisition quadrant during the retention probe trial. The observation that inactive and vehicle-treated animals had more difficulty during the first day of reacquisition, and also spent the most time in the acquisition quadrant during the retention probe trial, suggests that these groups of animals fail to alter their behavior to consistently succeed in the task.

Taken together, the anatomical findings and the behavioral findings suggest that voluntary exercise or agonism of HIF-1α result in cognitive facilitation. Although we cannot conclude that HIF-1α itself is a mediator of exercise-induced enhancement in learning, our findings do suggest that the downstream processes stimulated by increased HIF-1α, namely angiogenesis, are important mediators of alterations in behavioral performance.

General Conclusions and Future Directions

In conclusion, this dissertation has shown that a single bout of exercise rapidly increases the expression of HIF-1α in the hippocampus of adult rats, suggesting that at the start of an exercise regimen, the brain experiences a transient state of hypoxia. These

findings may also explain the initial apoptosis, and subsequent angiogenesis observed shortly after the start of an exercise regimen (Kerr & Swain, 2011). Our studies have also shown that alterations in vasculature, a downstream result of HIF-1α activation, mediate improved performance in a spatial learning task. These findings corroborate previous research that has shown that disruption of angiogenesis results in significant cognitive deficits (Kerr et al., 2010).

The current research has practical applications in both healthy and clinical populations. Exercise has been shown to contribute to enhanced cognitive performance in healthy human populations (Erickson et al., 2011; Hillman et al., 2003, 2006; Prakash et al., 2011; Winter et al., 2007). Additionally, a great deal of research has highlighted the neuroprotective effects of exercise if engaged in before or after stroke (Ding et al., 2003, 2004a, 2004b; Ke et al., 2011; Matsuda et al., 2011), and following brain injury (Griesbach et al., 2004, 2007). The findings of this dissertation add to this commentary by identifying the molecular and physiological processes activated by exercise, and how those processes in turn, relate to cognitive function. With this knowledge future research could investigate the effectiveness of treatments targeted at manipulation of HIF-1α expression and angiogenesis, via exercise or drug treatment, in brain injured populations. A particularly interesting study would be to combine exercise with drug treatment to determine if these processes work together to enhance cognitive performance in these clinical populations. Moreover, if future research findings suggest a direct relationship between the expression of HIF-1α and improved cognitive functioning, then in cases where exercise is not possible due to the severity of the brain insult or other physical

ailment, drug treatment to increase the expression of HIF-1α could be considered as a possible therapy to enhance cognitive performance.

Although this data answers a few of the questions that were asked regarding the immediate responses of exercise in the brain, more questions remain. We were unable to conclusively show that increased HIF-1α expression itself is involved in cognitive facilitation. An interesting follow-up to the current study would be to compare animals treated with either a HIF-1α antagonist or a HIF-1α agonist and assess behavioral performance using a more challenging paradigm (two trials per day in the MWM, for example) that may be more sensitive to treatment-induced changes in visuospatial learning performance. It is our contention that exercise increases HIF-1α expression and later causes angiogenesis, examination of the intermediate steps between increased HIF-1α levels and angiogenesis, namely the expression of VEGF and its high-affinity receptors, would also be an important study.

We have demonstrated that in the short-term, exercise causes a transient state of hypoxia in the brain, and Kerr and Swain (2011) found that exercise causes apoptosis shortly after commencement of physical activity. Although the resulting decreased brain oxygenation and apoptosis suggest that exercise may initially be detrimental to the brain, the subsequent exercise-induced alterations to brain morphology and physiology appear to compensate for and outweigh these seemingly damaging processes in the long-term. It is important to continue to evaluate the brain's response to exercise and to further elucidate the molecular mechanisms responsible for the significant improvements in cognitive functioning in exercising organisms. Exercise research is relevant across a

broad spectrum of academic disciplines, and more importantly, research of this type has countless practical applications in non-clinical and clinical populations alike.

References

Acker, T., & Acker, A. (2004). Cellular oxygen sensing need in CNS function: Physiological and pathological implications. *Journal of Experimental Biology, 207*, 3171-3188. doi: 10.1242/jeb.01075

Adamcio, B., Sargin, D., Stradomska, A., Medrihan, L., Gertler, C., Theis, F.,…Ehrenreich, H. (2008). Erythropoietin enhances hippocampal long-term potentiation and memory. *BMC Biology, 6*, 37-52. doi: 10.1186/1741-7007-6-37

Adamcio, B., Sperling, S., Hagemeyer, S., Walkingshaw, G., & Ehrenreich, H. (2010). Hypoxia inducible factor stabilization leads to lasting improvement of hippocampal memory in healthy mice. *Behavioural Brain Research, 208*, 80-84. doi: 10.1016/j.bbr.2009.11.010

Adkins, D. L., Boychuk, J., Rempel, M. S., & Kleim, J. A. (2006). Motor training induces experience-specific patterns of plasticity across motor cortex and spinal cord. *Journal of Applied Physiology,101*, 1776-1782. doi: 10.1152/japplphysiol.00515.2006

Aguiar, A. S., Tuon, T., Pinho, C. O., Silva, L. A., Andreazza, A. C., Kapczinski, F., Quevedo, J.,…Pinho, R. A. (2008). Intense exercise induces mitochondrial dysfunction in mice brain. *Neurochemical Research, 33*, 51-58. doi: 10.1007/s11064-007-9406-x

Altman, J., & Das, G. D. (1967). Postnatal neurogenesis in the guinea pig. *Nature, 214*(5093), 1098-1101.

Amaral, S. L., Sanchez, L. S., Chang, A. J. B. A., Rossini, L. V., & Michelini, L. C.

(2008). Time course of training-induced microcirculatory changes and of VEGF expression in skeletal muscles of spontaneously hypertensive female rats. *Brazilian Journal of Medical and Biological Research, 41*, 424-431.

Ameln, H., Gustafsson, T., Sundberg, C. J., Okamoto, K., Jansson, E., Poellinger, L., & Makino, Y. (2005). Physiological activation of hypoxia-inducible factor-1 in human skeletal muscle. *FASEB Journal, 19*(8), 1009-1011. doi: 10.1096/fj.04-2304-fje

Ang, E., Dawe, G. S., Wong, P. T. H., Moochhala, S., & Ng, Y. (2006). Alterations in spatial learning and memory after forced exercise. *Brain Research, 1113*, 186-193.

Beck, T., & Krieglstein, J. (1987). Cerebral circulation, metabolism, and blood-brain barrier of rats in hypercapnic hypoxia. *American Journal of Physiology, 252*(3), H504-512.

Bednarczyk, M. R., Aumont, A., Decary, S., Bergeron, R., & Fernandes, K. J. L. (2009). Prolonged voluntary wheel-running stimulates neural precursors in the hippocampus and forebrain of adult CD1 mice. *Hippocampus, 19*, 913-927. doi: 10.1002/hipo.20621

Benveniste, H., Jorgensen, M. B., Diemer, N. H., & Hansen, A. J. (1988). Calcium accumulation by glutamate receptor activation is involved in hippocampal cell damage after ischemia. *Acta Neurologica Scandinavica, 78*, 529-536.

Benyo, D. F., Ravindranath, N., Bassett, S., Hutchison, J., & Zeleznik, A.J. (1993). Cellular aspects of corpus luteum formation in the primate. *Human Reproduction, 8*(supplement 2), 102-106.

Berchtold, N. C., Costello, N., & Cotman, C. W. (2010). Exercise and time-dependent benefits to learning and memory. *Neuroscience, 167*, 588-597. doi: 10.1016/j.neuroscience.2010.02.050

Bergeron, M., Yu, A. Y., Solway, K. E., Semenza, G. L., & Sharp, F. R. (1999). Induction of hypoxia-inducible factor-1 (HIF-1) and its target genes following focal ischaemia in rat brain. *European Journal of Neuroscience, 11*, 4159-4170.

Bernaudin, M., Nedelec, A., Divoux, D., MacKenzie, E. T., Petit, E., Schumann-Bard, P. (2002). Normobaric hypoxia induces tolerance to focal permanent cerebral ischemia in association with increased expression of hypoxia-inducible factor-1 and its target genes, erythropoietin and VEGF, in the adult mouse brain. *Journal of Cerebral Blood Flow and Metabolism, 22*, 393-403.

Bikfalvi A., & Bicknell, R. (2002). Recent advances in angiogenesis, anti-angiogenesis and vascular targeting. *TRENDS in Pharmacological Science, 23*(12), 576-582.

Birot, O. J. B., Koulmann, N., Peinnequin, A., & Bigard, X. A. (2003). Exercise-induced expression of vascular endothelial growth factor mRNA in rat skeletal muscle is dependent on fibre type. *Journal of Physiology, 552*(1), 213-221. doi: 10.1113/jphysiol.2003.043026

Black, J. E., Isaacs, K. R., Anderson, B. J., Alcantara, A. A., & Greenough, W. T. (1990). Learning causes synaptogenesis, whereas motor activity causes angiogenesis, in cerebellar cortex of adult rats. *Proceedings of the National Academy of Sciences of the USA, 87*, 5568-5572.

Bonnekoh, P., Barbier, A., Oschlies, U., & Hossmann, K. A. (1990). Selectie vulnerability in the gerbil hippocampus: Morphological changes after 5-min ischemia and long survival times. *Acta Neuropathologica, 80*, 18-25.

Boreo, J. A., Ascher, J., Arregui, A., Rovainen, C., & Woolsey, T. A. (1999). Increased brain capillaries in chronic hypoxia. *Journal of Applied Physiology, 86*, 1211-1219.

Brines, M., & Cerami, A. (2005). Emerging biological roles for erythropoietin in the nervous system. *Nature Reviews Neuroscience, 6*, 484-494.

Bruel-Jungerman, E., Veyrac, A., Dufour, F., Horwood, J., Laroche, S., & Davis, S. (2009). Inhibition of PI3K-Akt signaling blocks exercise-mediated enhancement of adult neurogenesis and synaptic plasticity in the dentate gyrus. *PLOS One, 4*(11), e7901. doi: 10.1371/journal.pone.0007901

Bulinski, S. C., Thompson, K. J., Powell, S. K., Sikorski, A. M., & Swain, R. A. (2000, November). *Increased immunolabeling of Flk-1 receptors in primary motor cortex of the adult rat following exercise.* Poster session presented at the Society for Neuroscience, New Orleans.

Caley, D. W., & Maxwell, D. S. (1970). Development of the blood vessels and extracellular spaces during postnatal maturation of rat cerebral cortex. *Journal of Comparative Neurology, 138*, 31-48.

Cantarella, G., Lempereur, L., Presta, M., Ribatti, D., Lombardo, G., Lazarovici, P., Zappala, G.,…Bernardini, R. (2002). Nerve growth factor-endothelial cell interaction leads to angiogensis *in vitro* and *in vivo*. *The FASEB Journal, 16*, 1307-1309. doi: 10.1096/fj.01-1000fje

Carlson, N. R. (2007). *Physiology of Behavior* (9th edition). Boston: Pearson.

Carraro, U., & Franceschi, C. (1997). Apoptosis of skeletal and cardiac muscles and physical exercise. *Aging, Clinical, and Experimental Research, 9,* 19-34.

Chae, C., & Kim, H. (2009). Forced, moderate-intensity treadmill exercise suppresses apoptosis by increasing the level of NGF and stimulating phosphatidylinositol 3-kinase signaling in the hippocampus of induced aging rats. *Neurochemistry International, 55*, 208-213. doi: 10.1016/j.neuint.2009.02.024

Chavez, J. C., Agani, F., Pichuile, P., & LaManna, J. (2000). Expression of hypoxia -inducible factor-1α in the brain of rats during chronic hypoxia. *Journal of Applied Physiology, 89*, 1937-1942.

Chavez, J. C., & LaManna, J. C. (2002). Activation of hypoxia-inducible factor-1 in the rat cerebral cortex after transient global ischemia: Potential role of insulin-like growth factor-1. *The Journal of Neuroscience, 22*(20), 8922-8931.

Chen, L. Endler, A., & Shibasaki, F. (2009). Hypoxia and angiogenesis: Regulation of hypoxia-inducible factor via novel binding factors. *Experimental and Molecular Medicine, 41*(12), 849-857. doi: 10.3858/emm.2009.41.12.103

Chinsomboon, J., Ruas, J., Gupta, R. K., Thom, R., Shoag, J., Rowe, G. C., Swada, N.,…Arany, Z. (2009). The transcriptional coactivatorPGC-1α mediates exercise-induced angiogenesis in skeletal muscle. *PNAS, 106*(50), 21401-21406. doi: 10.1073/pnas.0909131106

Choi, D. W. (1992). Excitotoxic cell death. *Journal of Neurobiology, 23*(9), 1261-1276.

Chu, P. W. Y., Beart, P. M., & Jones, N. M. (2010). Preconditioning protects against

oxidative injury involving hypoxia-inducible factor-1 and vascular endothelial growth factor in cultured astrocytes. *European Journal of Pharmacology, 633*, 24-32. doi: 10.1016/j.ejphar.2010.02.008

Contartaze, R. V. L., Manchado, F. D. B., Gobatto, C. A., & de Mello, M. A. R. (2008). Stress biomarkers in rats submitted to swimming and treadmill exercises. *Comparative Biochemistry and Physiology, Part A, 151*, 415-422.

Couillard-Despres, S., Winner, B., Schaubeck, S., Aigner, R., Vroemen, M., Weidner, N., Bogdahn, U.,…Aigner, L. (2005). Doublecortin expression levels in adult brain reflect neurogenesis. *European Journal of Neuroscience, 21*, 1-14. doi: 10.1111/j.1460-9568.2004.03813.x

Dawson, D. A., Graham, D. I., McCulloch, J., & Macrae, I. M. (1994). Anti-ischaemic efficacy of a nitric oxide synthase inhibitor and a N-methyl-D-aspartate receptor antagonist in models of transient and permanent focal cerebral ischaemia. *British Journal of Pharmacology, 113*, 247-253.

Dietrich, M. O., Andrews, Z. B., & Horvath, T. L. (2008). Exercise-induced synaptogenesis in the hippocampus is dependent upon UCP2-regulated mitochondrial adaptation. *The Journal of Neuroscience, 28*(42), 10766-10771. doi: 10.1523/JNEUROSCI.2744-08.2008

Ding, Y., Li, J., Clark, J., Diaz, F. G., & Rafols, J. A. (2003). Synaptic plasticity in the thalamic nuclei enhanced by motor skill training in rat with transient middle cerebral artery occlusion. *Neurological Research, 25*, 189-194.

Ding, Y., Li, J., Lai, Q., Azam, S., Rafols, J. A., & Diaz, F. G. (2002). Functional

improvement after motor training is correlated with synaptic plasticity in rat thalamus. *Neurological Research, 24*, 829-836.

Ding, Y., Li, J., Luan, X., Ding, H., Lai, Q., Rafols, J. A.,…Diaz, F. G. (2004a). Exercise pre-conditioning reduces brain damage in ischemic rats that may be associated with regional angiogenesis and cellular overexpression of neurotrophin. *Neuroscience, 124*, 583-591. doi: 10.1016/j.neuroscience.2003.12.029

Ding, Y. H., Li, J., Zhao, Y., Rafols, J. A., Clarck, J. C., & Ding, Y. (2006a). Cerebral angiogenesis and expression of angiogenic factors in aging rats after exercise. *Current Neurovascular Research, 3*, 15-23.

Ding, Y., Luan, X., Li, J., Rafols, J. A., Guthinkonda, M., Diaz, F. G., & Ding, Y. (2004b). Exercise-induced overexpression of angiogenic factors and reduction of ischemia/reperfusion injury in stroke. *Current Neurovascular Research, 1*, 411-420.

Ding, Q., Vaynman, S., Akhavan, M., Ying, Z., & Gomez-Pinilla, F. (2006c). Insulin-like growth factor I interfaces with brain-derived neurotrophic factor-mediate synaptic plasticity to modulate aspects of exercise-induced cognitive function. *Neuroscience, 140*, 823-833. doi: 10.1016/j.neuroscience.2006.02.084

Ding, Q., Vaynman, S., Souda, P., Whitelegge, J. P., & Gomez-Pinilla, F. (2006b). Exercise affects energy metabolism and neural plasticity-related proteins in the hippocampus as revealed by proteomic analysis. *European Journal of Neuroscience, 24*, 1265-1276. doi: 10.1111/j.1460-9568.2006.05026.x

Dunn, J. F., Grinberg, O., Roche, M., Nwaigwe, C. I., Hou, H. G., & Swartz, H. M.

(2000). Noninvasive assessment of cerebral oxygenation during acclimation to hypobaric hypoxia. *Journal of Cerebral Blood Flow and Metabolism, 2*0, 1632-1635.

During, M. J., & Cao, L. (2006). VEGF, a mediator of the effect of experience on hippocampal neurogenesis. *Current Alzheimer Research, 3*, 29-33.

Ekstrand, J., Hellsten, J., & Tingstrom, A. (2008). Environmental enrichment, exercise and corticosterone affect endothelial cell proliferation in adult rat hippocampus and prefrontal cortex. *Neuroscience Letters, 442*, 203-207. doi: 10.1016/j.neulet.2008.06.085

El-Kordi, A., Radyushkin, K., & Ehrenreich, H. (2009). Erythropoietin improves operant conditioning and stability of cognitive performance in mice. *BMC Biology, 7*, 37-44. doi: 10.1186/1741-7007-7-37

Ema, M., Taya, S., Yokotani, N., Sogawa, K., Matsuda, Y., & Fujii-Kuriyama, Y. (1997). A novel bHLH-PAS factor with close sequence similarity to hypoxia-inducible factor 1α regulates the VEGF expression and is potentially involved in lung and vascular development. *Proceedings of the National Academy of the United States of America, 94*, 4273-4278.

Emanueli, C., Salis, M. B., Pinna, A., Graiani, G., Manni, L., & Madeddu, P. (2002). Nerve growth factor promotes angiogenesis and arteriogenesis in ischemic hindlimbs. *Circulation, 106*, 2257-2262. doi: 10.1161/01.CIR.0000033971.56802.C5

Erickson, K. I., Voss, M.W., Prakash, R. S., Basak, C., Szabo, A., Chaddock, L., Kim, J.

S.,…Kramer, A.F. (2011). Exercise training increases size of hippocampus and improves memory. *PNAS, 108*(7), 3017-3022. doi: 10.1073/pnas.1015950108

Eriksen, B. A., & Eriksen, C. W. (1974). Effects of noise letters upon the identification of a target letter in a nonsearch task. *Perception & Psychophysics, 16*(1), 143-149.

Fabel, K., Fabel, K., Tam, B., Kaufer, D., Baiker, A., Simmons, N.,…Palmer, T. D. (2003). VEGF is necessary for exercise-induced adult hippocampal neurogenesis. *European Journal of Neuroscience, 18*, 2803-2812. doi: 10.1046/j.1406-9568.2003.03041.x

Fabel, K., Wolf, S. A., Ehninger, D., Babu, H., Leal-Galicia, P., & Kempermann, G. (2009). Additive effects of physical exercise and environmental enrichment on adult hippocamal neurogenesis in mice. *Frontiers in Neurogenesis, 1*, 1-7. doi: 10.3389/neuro.22.002.2009

Farmer, J., Zhao, X., van Praag, H., Wodtke, K., Gage, F. H., Christie, B. R. (2004). Effects of voluntary exercise on synaptic plasticity and gene expression in the dentate gyrus of adult male Sprague-Dawley rats *in vivo*. *Neuroscience, 124*, 71-79. doi: 10.1016/j.neuroscience.2003.09.029

Fawcett, J. W., Rosser, A. E., & Dunnett, S. B. (2001). *Brain Damage, Brain Repair*. New York: Oxford University Press.

Ferrara, N. (1999). Vascular endothelial growth factor: molecular and biological aspects. *Current Topics in Microbiology and Immunology, 237*, 1-29.

Ferrara, N. (2004). Vascular endothelial growth factor: Basic science and clinical progress. *Endocrine Reviews, 25*(4), 584-611. doi: 10.1210/er.2003-0027

Ferrara, N., Chen, H., Davis-Smyth, T., Gerber, H., Nguyen, T., Peers., D., Chisholm.

V.,…Schwall, R.H. (1998). Vascular endothelial growth factor is essential for corpus luteum angiogenesis. *Nature Medicine, 4*(3), 336-340.

Ferrara, N., & Davis-Smyth, T. (1997). The biology of vascular endothelial growth factor. *Endocrine Reviews, 18*(1), 4-25.

Folkman, J. (1995). Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nature Medicine, 1*(1), 27-31.

Folkman, J., Watson, K., Ingber, D., & Hanahan, D. (1989). Induction of angiogenesis during the transition from hyperplasia to neoplasia. *Nature, 339*, 58-61.

Fong, G. (2009). Regulation of angiogenesis by oxygen sensing mechanisms. *Journal of Molecular Medicine, 87*, 549-560. doi: 10.1007/s00109-009-0458-z

Fong, G., Rossant, J., Gertsenstein, M., & Breitman, M. L. (1995). Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of the vascular endothelium. *Nature, 376*, 66-70.

Fraser, H. M., Dickson, S. E., Lunn, S. F., Wulff, C., Morris, K. D., Carroll, V. A., & Bicknell, R. (2000). Suppression of luteal angiogenesis in the primate after neutralization of vascular endothelial growth factor. *Endocrinology, 141*, 995-1000. doi: 10.1210/en.141.3.995

Fraser, H. M., & Wulff, C. (2003). Angiogenesis in the corpus luteum. *Reproductive Biology and Endocrinology, 1*, 1-8. Retrieved from http://www.rbej.com/content/1/1/88

Fujisawa, H., Dawson, D., Browne, S. E., MacKay, K. B., Bullock, R., & McCulloch. (1993). Pharmacological modification of glutamate neurotoxicity in vivo. *Brain Research, 629*, 73-78.

Gavin, T. P., Drew, J. L., Kubik, C. J., Pofahl, W. E., & Hickner, R. C. (2007a). Acute resistance exercise increases skeletal muscle angiogenic growth factor expression. *Acta Physiologica, 191*, 139-146. doi: 10.1111/j.1748-1716.2007.01723.x

Gavin, T. P., Ruster, R. S., Carrithers, J. A., Zwetsloot, K. A., Kraus, R. M., Evans, C. A.,…Hickner, R. C. (2007b). No difference in the skeletal muscle angiogenic response to aerobic exercise training between young and aged men. *Journal of Physiology, 585*(1), 231-239. doi: 10.1113/jphysiol.2007.143198

Gavin, T. P., Westerkamp, L. M., & Zwetsloot, K. A. (2006). Soleus, plantaris and gastrocnemius VEGF mRNA reseponse to hypoxia and exercise are preserved in aged compared with young female C57BL/6 mice. *Acta Physiologica, 188*, 113-121. doi: 10.1111/j.1748-1716.2006.01609.x

Geng, T., Li, P., Okutsu, M., Yin, X., Kwek, J., Zhang, M., & Yan, Z. (2010). PGC-1α plays a functional role in exercise-induced mitochondrial biogenesis and angiogenesis but not fiber-type transformation in mouse skeletal muscle. *American Journal of Physiology: Cell Physiology, 298*, C572-C579. doi: 10.1152/ajpcell.00481.2009

Gidday, J. M., Fitzgibbons, J. C., Shah, A. R., & Park, T. S. (1994). Neuroprotection from ischemic brain injury by hypoxic preconditioning in the neonatal rat. *Neuroscience Letters, 168*, 221-224.

Gomez-Pinilla, F., Vaynman, S., & Ying, Z. (2008). Brain-derived neurotrophic factor functions as a metabotropin to mediate the effects of exercise on cognition. *European Journal of Neuroscience, 28*, 2278-2287. doi: 10.1111/j.1460-9568.2008.06524.x

Gomez-Pinilla, F., Ying, Z., Roy, R. R., Molteni, R., & Edgerton, R. (2002). Voluntary exercise induces a BDNF-mediated mechanism that promotes neuroplasticity. *Journal of Neurophysiology, 88*, 2187-2195. doi: 10.1152/jn.00152.2002

Gould, E., & Gross, C. G. (2002). Neurogenesis in adult mammals: Some progress and some problems. *The Journal of Neuroscience, 22*(3), 619-623.

Griesbach, G. S., Gomez-Pinilla, F., & Hovda, D. A. (2007). Time window for voluntary exercise-induced increases in hippocampal neuroplasticity molecules after traumatic brain injury is severity dependent. *Journal of Neurotrauma, 24*(7), 1161-1171. doi: 10.1089/neu.2006.0255

Griesbach, G. S., Hovda, D. A., & Gomez-Pinilla, F. (2009). Exercise-induced improvement in cognitive performance after traumatic brain-injury in rats is dependent upon BDNF activation. *Brain Research, 1288,* 105-115. doi: 10.1016/j.brainres.2009.06.045

Griesbach, G. S., Hovda, D. A., Molteni, R., Wu, A., & Gomez-Pinilla, F. (2004). Voluntary exercise following traumatic brain injury: Brain-derived neurotrophic factor upregulation and recovery of function. *Neuroscience, 125*, 129-139. doi: 10.1016/j.neuroscience.2004.01.030

Griesemer, D., & Mautes, A. M. (2007). Closed head injury causes hyperexcitability in rat hippocampal CA1 but not CA3 pyramidal cells. *Journal of Neurotrauma, 24*(12), 1823-1832.

Haege, S., Galetzka, D., Zechner, U., Haaf, T., Gamerdinger, M., Behl, C., Hiemke, C., & Schmitt, U. (2010). Spatial lerning and expression patterns of PP1 mRNA in mouse hippocampus. *Neuropsychobiology, 61*, 188-196. doi: 10.1159/000297736

Hamburger, V. (1993). The history and discovery of the nerve growth factor. *Journal of Neurobiology, 24*(7), 893-897.

Hamrick, S. E. G., McQuillen, P. S., Jiang, X., Mu, D., Madan, A., & Ferriero, D. M. (2005). A role for hypoxia-inducible factor-1α in desferoxamine neuroprotection. *Neuroscience Letters, 379*, 96-100. doi: 10.1016/j.neulet.2004.12.080

Harik, N., Harik, S. I., Kuo, N., Sakai, K., Przybylski, R. J., & LaManna, J. C. (1996). Time-course and reversibility of hypoxia-induced alterations in cerebral vascularity and cerebral capillary glucose transporter density. *Brain Research, 737*, 335-338.

Harik, S. I., Hritz, M. A., & LaManna, J. C. (1995). Hypoxia-induced brain angiogenesis in the adult rat. *Journal of Physiology, 485*(2), 525-530.

Harten, S. K., Ashcroft, M., & Maxwell, P. H. (2010). Prolyl hydroxylase domain inhibitors: A route to HIF activation and neuroprotection. *Antioxidants & Redox Signaling, 12*(4), 459-480. doi: 10.1089/ars.2009.2870

Hawkins, H. L., Kramer, A. F., & Capaldi, D. (1992). Aging, exercise, and attention. *Psychology and Aging, 7*(4), 643-653.

Heron, M., Hoyert, D. L., Murphy, S. L., Xu, J., Kochanek, K. D., & Tejada-Vera, B. (2009). Deaths: Final data for 1006. *National Vital Statistics Reports, 57*(14). U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, National Center for Health Statistics, National Vital Statistics Section: Washington, D.C.

Hillman, C. H., Motl, R. W., Pontifex, M. B., Posthuma, D., Stubbe, J. H., Boomsma, D.

I., & de Gues, E. J. C. (2006). Physical activity and cognitive function in a cross-section of younger and older community-dwelling individuals. *Health Psychology, 25*(6), 678-687. doi: 10.1037/0278-6133.25.6.678

Hillman, C. H., Snook, C. M., & Jerome, G. J. (2003). Acute cardiovascular exercise and executive control function. *International Journal of Psychophysiology, 48*, 307-314. doi: 10.1016/S0167-8760(03)00080-1

Howard, C. V., & Reed, M. G. (1998). *Unbiased stereology: Three-Dimensional Measurement in Microscopy*. New York: Springer.

Hu, Y., Wang, Y., Guo, T., Wei, W., Sun, C., Zhang, L., & Huang, J. (2007). Identification of brain-derived neurotrophic factor as a novel angiogenic protein in multiple myeloma. *Cancer Genetics and Cytogenetics, 178*, 1-10. doi: 10.1016/j.cancergencyto.2007.05.028

Hu, S., Ying, Z., Gomez-Pinilla, F., & Frautschy, S. A. (2009). Exercise can increase small heat shock proteins (sHSP) and pre- and post-synaptic proteins in the hippocampus. *Brian Research, 1249*, 191-201. doi: 10.1016/j.brainres.2008.10.054

Huang, A. M., Jen, C. J., Chen, H. F., Yu, L., Kuo, Y. M., & Chen, H. I. (2006). Compulsive exercise acutely upregulates rat hippocampal brain-derived neurotrophic factor. *Journal of Neural Transmission, 113*, 803-811. doi: 10.1007/s00702-005-0359-4

Isaacs, K. R., Anderson, B. J., Alcantara, A. A., Black, J. E., & Greenough, W. T. (1992).

Exercise and the brain: Angiogenesis in the adult rat cerebellum after vigorous physical activity and motor skill learning. *Journal of Cerebral Blood Flow and Metabolism, 12*, 110-119.

Jewell, U.R., Kvietikova, I, Scheid, A., Bauer, C., Wenger, R.H., & Gassman, M. (2001). Induction of HIF-1α in response to hypoxia is instantaneous. *FASEB Journal, 15*, 1312-1314. doi: 10.1096/fj.00-0732fje

Jiang, B. H., Semenza, G. L., Bauer, C., & Marti, H. H. (1996). Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O2 tension. *American Journal of Physiology, 271*(4), C1172-C1180.

Jiang, B., Zheng, J. Z., Leung, S. W., Roe, R., & Semenza, G. L. (1997). Transactivation and inhibitory domains of hypoxia-inducible factor 1α. *Journal of Biological Chemistry, 272*(31), 19253-19260.

Jones, N. M., & Bergeron, M. (2001). Hypoxic preconditioning induces changes in HIF-1 target genes in neonatal rat brain. *Journal of Cerebral Blood Flow and Metabolism, 21*, 1105-1114.

Kaelin, W. G., & Ratcliffe, P. J. (2008). Oxygen sensing by metazoans: The central role of the HIF hydroxylase pathway. *Molecular Cell, 30*, 393-402. doi: 10.1016/j.molcel.2008.04.009

Kandell, E. R., Schwartz, J. H., & Jessell, T. M. (2000). *Principles of Neural Science* (4th edition). New York: McGraw-Hill.

Kaplan, M. S., & Bell, D. H. (1984). Mitotic neuroblasts in the 9-day-old and 11-month -old rodent hippocampus. *The Journal of Neuroscience, 4*(6), 1429-1441.

Kassahn, K. S., Crozier, R. H., Portner, H. O., & Caley, M. J. (2009). Animal

performance and stress: Responses and tolerance limits at different levels of biological organisation. *Biological Reviews, 84*, 277-292. doi: 10.1111/j.1469-185X.2008.00073.x

Kasiganesan, H., Sridharan, V., & Wright, G. (2007). Prolyl hydroxylase inhibitor treatment confers whole-animal hypoxia tolerance. *Acta Physiologica, 190*, 163-169. doi: 10.1111/j.1748-1716.2007.01676.x

Kawanami, D., Mahabeleshwar, G. H., Lin, Z., Atkins, G. B., Hamik, A., Haldar, S. M.,…Jain, M. K. (2009). Kruppel-like factor 2 inhibits hypoxia-inducible factor 1α expression and function in the endothelium. *Journal of Biological Chemistry, 284*(31), 20522-20530. doi: 10.1074/jbc.M109.025346

Kawasaki, K., Traynelis, S. F., & Dingledine, R. (1990). Different response of CA1 and CA3 regions to hypoxia in rat hippocampal slice. Journal of Neurophysiology, 63(3), 385-394.

Kempermann, G., Gast, D., Kronenberg, G., Yamaguchi, M., & Gage, F. H. (2003). Early determination and long-term persistence of adult-generated new neurons in the hippocampus of mice. *Development, 130*, 391-399. doi: 10.1242/dev.00203

Kempermann, G., Jessberger, S., Steiner, B., & Kronenberg, G. (2004). Milestones of neuronal development in the adult hippocampus. *Trends in Neuroscience, 27*(8), 447-452.

Kempermann, G., Kuhn, H. G., & Gage, F. H. (1998). Experience-induced neurogenesis in the senescent dentate gyrus. *The Journal of Neuroscience, 18*(9), 3206-3212.

Ke, Z., Yip, S. P., Li, L., Zheng, X. X., & Tong, K. Y. (2011). The effects of voluntary,

involuntary, and forced exercises on brain-derived neurotrophic factor and motor function recovery: A rat brain ischemia model. *PLoS One, 6*(2), e16643. doi: 10.1371/journal.pone.0016643.

Kerr, A. L., Steuer, E. L., Pochtarev, V., & Swain, R. A. (2010). Angiogenesis but not neurogenesis is critical for normal learning and memory acquisition. *Neuroscience, 171*, 214-226. doi: 10.1016/j.neuroscience.2010.08.008

Kerr, A. L., & Swain, R. A. (2011). Rapid cellular genesis and apoptosis: Effects of exercise in the adult rat. *Behavioral Neuroscience, 125*(1), 1-9. doi: 10.1037/a0022332

Khorshidahmad, T., Tabrizian, K., Vakilzadeh, G., Nikbin, P., Moradi, S., Hosseini -Sharifabad, A., Roghani, A., …Sharifzadeh, M. (2012). Interactive effects of a protein kinase AII inhibitor and testosterone on spatial learning in the Morris Water Maze. *Behavioural Brain Research, 228*, 432-439. doi: 10.1016/j.bbr.2011.12.028

Kinni, H., Guo, M., Ding, J. Y., Konakondla, S., Dornbos, D., Tran, R.,…Ding, Y. (2011). Cerebral metabolism after forced or voluntary physical exercise. *Brain Research, 1388*, 48-55. doi: 10.1016/j.brainres.2011.02.076

Kim, S., Kim, H., Jang, M., Lim, B., Kim, Y., Kim, Y. P., Kim, S. S.,…Kim, C. J. (2002). Treadmill exercise increases cell proliferation without altering of apoptosis in dentate gyrus of Sprague-Dawley rats. *Life Sciences, 71*, 1331-1340.

Kim, S. E., Ko, I., Kim, B. K., Shin, M., Cho, S., Kim, C. G.,…Jee, Y. (2010). Treadmill

exercise prevents aging-induced failure of memory through an increase in neurogenesis and suppression of apoptosis in rat hippocampus. *Experimental Gerontology, 45*, 357-365. doi: 10.1016/j.exger.2010.02.005

Kirino, T., Tsujita, Y., & Tamura, A. (1991). Induced tolerance to ischemia in gerbil hippocampal neurons. *Journal of Cerebral Blood Flow and Metabolism, 11*, 299 -307.

Kivela, R., Silvennoinen, M., Lehti, M., Jalava, S., Vihko, V., & Kainulainen, H. et al. (2008). Exercise-induced expression of angiogenic growth factors in skeletal muscle and in capillaries of healthy and diabetic mice. *Cardiovascular Diabetology, 7*, 13-22. doi: 10.1186/1475-2840-7-13

Kleim, J. A., Cooper, N. R., & Vandenberg, P. M. (2002). Exercise induces angiogenesis but does not alter movement representations within rat motor cortex. *Brain Research, 934*, 1-6.

Klintsova, A. Y., Dickson, E., Yoshida, R. & Greenough, W. T. (2004). Altered expression of BDNF and its high-affinity receptor TrkB in response to complex motor learning and moderate exercise. *Brain Research, 1028*, 92-104. doi: 10.1016/j.brainres.2004.09.003

Koko, V., Djordjeviae, J., Cvijiae, G., & Davidoviae, V. (2007). Effect of acute heat stress on rat adrenal glands: a morphological and stereological study. *Journal of Experimental Biology, 207*, 4225-4230. doi: 10.1242/jeb.01280

Kreisman, N. R., & LaManna, J. C. (1999). Rapid and slow swelling during hypoxia in the CA1 region of rat hippocampal slices. *Journal of Neurophysiology, 82*, 320-329.

Kreisman, N. R., Soliman, S., & Gozal, D. (2000). Regional differences in hypoxic depolarization and swelling in hippocampal slices. *Journal of Neurophysiology, 83*, 1031-1038.

LaManna, J. C., Chavez, J. C., & Pichiule, P. (2004). Structural and functional adaptation to hypoxia in the rat brain. *Journal of Experimental Biology, 207*, 3163-3169. doi: 10.1242/jeb.00976

LaManna, J. C., Vendel, L. M., & Farrell, R. M. (1992). Brain adaptation to chronic hypobaric hypoxia in rats. *Journal of Applied Physiology, 72*(6), 2238-2243.

Larrivee, B., & Karsan, A. (2000). Signaling pathways induced by vascular endothelial growth factor. *International Journal of Molecular Medicine, 5*, 447-456.

Laufs, U., Werner, N., Link, A., Endres, M., Wassmann, S., Jurgens, K., Miche, E….Nickenig, G. (2004). Physical training increases endothelial progenitor cells, inhibits neointima formation, and enhances angiogenesis. *Circulation, 109*, 220-226. doi: 10.1161/01.CIR.0000109141.48980.37

Lauro, K. L., & LaManna, J. C. (1997). Adequacy of cerebral vascular remodeling following three weeks of hypobaric hypoxia. *Oxygen Transport to Tissue XVIII, 411*(18), 369-376.

Lee, H. S., Han, J., Bai, H., & Kim, K. (2009). Brain angiogenesis in developmental and pathological processes: regulation, molecular and cellular communication at the neurovascular interface. *The FEBS Journal, 276*, 4622-4635. doi: 10.1111/j.1742-4658.2009.07174.x

Lewin, B. (2008). *Genes IX*. Boston: Jones and Bartlett Publishers.

Li, Z., Bao, S., Wu, Q., Wang, H., Eyler, C., Sathomsumetee, S.,…Rich, J. N. (2009).

Hypoxic-inducible factors regulate tumorigenic capacity of glioma stem cells. *Cancer Cell, 15*, 501-513. doi: 10.1016/j.ccr.2009.03.018

Li, L., Qu, Y., Li, J., Xiong, Y., Mao, M., & Mu, D. (2007). Relationship between HIF -1α expression and neuronal apoptosis in neonatal rats with hypoxia-ischemia brain injury. *Brain Research, 1180*, 133-139. doi: 10.1016/j.brainres.2007.08.059

Li, Y., Zhao, C., Calvert, J. W., Colohan, A. R. T., & Zhang, J. H. (2005). Multiple effects of hyperbaric oxygen on the expression of HIF-1α and apoptotic genes in a global ischemia-hypotension rat model. *Experimental Neurology, 191*, 198-201. doi: 10.1016/j.expneurol.2004.08.036

Lindeburg, M.R. (2006). *Civil Engineering Reference Manual: For the PE Exam* (10th ed.). Belmont, CA: Professional Publications, Inc.

Lloyd, P. G., Prior, B. M., Yeng, H. T., & Terjung, R. L. (2003). Angiogenic growth factor expression in rat skeletal muscle in response to exercise training. *American Journal of Physiology: Heart and Circulatory Physiology, 284*, H1668-H1678. doi: 10.1152/ajpheart.00743.2002

Lloyd-Jones, D., Adams, R., Carnethon, M., de Simone, G., Ferguson, B., Flegal, K.,…Hong, Y. (2009). Heart disease and stroke statistics – 2009 update: A report from the Amercian Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation, 119*, e21-e181. doi: 10.1161/CIRCULATIONAHA.108.191261

Lopez-Lopez, C., LeRoith, D., & Torres-Aleman, I. (2004). Insulin-like growth factor I is required for vessel remodeling in the adult brain. *PNAS, 101*(26), 9833-9838. doi: 10.1073/pnas.0400337101

Lou, S., Liu, J., Chang, H., & Chen, P. (2008). Hippocampal neurogenesis and gene expression depend on exercise intensity in juvenile rats. *Brain Research, 1210*, 48-55. doi: 10.1016/j.brainres.2008.02.080

Lu, L., Bao, G.., Chen, H., Xia, P., Fan, X., Zhang, J., Pei, G., & Ma, L. (2003). Modification of hippocampal neurogenesis and neuroplasticity by social environments. *Experimental Neurology, 183*, 600-609. doi: 10.1016/S0014-4886(03)00248-6

Lu, G., Ding, D., & Shi, M. (1999). Acute adaptation of mice to hypoxic hypoxia. *Biological Signals and Receptors, 8*, 247-255.

Lundby, C., Gassmann, M., & Pilegaard, H. (2006). Regular endurance training reduces HIF-1α and HIF-2α mRNA expression in human skeletal muscle in normoxic conditions. *European Journal of Applied Physiology, 96*, 363-369. doi: 10.1007/s00421-005-0085-5

Lushnikova, I., Orlovsky, M., Dosenko, V., Maistrenko, A., & Skibo, G. (2011). Brief anoxia preconditioning and HIF prolyl-hydroxylase inhibition enhances neuronal resistance in organotypic hippocampal slices on model of ischemic damage. *Brain Research, 1386*, 175-183. doi: 10.1016/j.brainres.2011.02.033

Makino, Y., Cao, R., Svensson, K., Bertilsson, G., Asman, M., Tanaka, H.,…Poellinger, L. (2001). Inhibitory PAS domain protein is a negative regulator of hypoxia-inducible gene expression. *Nature, 414*, 550-554.

Marais, L. Stein, D. J, & Daniels, W. M. U. (2009). Exercise increases BDNF levels in the striatum and decreases depressive-like behavior in chronically stressed rats. *Metabolic Brain Disease, 24*, 587-597. doi: 10.1007/s11011-009-9157-2

Marin, M. T., Cruz, F. C., & Palenta, C. S. (2007). Chronic restraint or variable stresses differently affect the behavior, corticosterone secretion and body weight in rats. *Physiology & Behavior, 90*, 29-35.

Marti, H. G. H., Bernaudin, M., Bellail, A., Schoch, H., Euler, M., Petit, E., & Risau, W. (2000). Hypoxia-induced vascular endothelial growth factor expression precedes neuovascularization after cerebral ischemia. *American Journal of Pathology, 156*(3), 965-976.

Matsuda, F., Sakakima, H., & Yoshida, Y. (2011). The effects of early exercise on brain damage and recovery after focal cerebral infarction in rats. *Acta Physiologica, 201*, 275-187.

Millauer, B., Wizigmann-Voos, S., Schnurch, H., Martinez, R., Moller, N. P. H., Risau, W., & Ullrich, A. (1993). High-affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. *Cell, 72*, 835-846.

Miller, B. A., Perez, R. S., Shah, A. R., Gonzales, E. R., Park, T. S., & Gidday, J. M. (2001). Cerebral protection by hypoxic preconditioning in a murine model of focal ischemia-reperfusion. *Neuroreport, 12*(8), 1663-1669.

Milosevic, J., Maisel, M., Wegner, F., Leuchtenberger, J., Wenger, R. H., Gerlach, M.,…Schwarz, J. (2007). Lack of hypoxia-inducible factor-1α impairs midbrain neural precursor cells involving vascular endothelial growth factor signaling. *The Journal of Neuroscience, 27*(2), 412-421. doi: 10.1523/JNEUROSCI.2482-06.2007

Mironov, V., Hritz, M. A., LaManna, J. C., Hudetz, A. G., & Harik, S. I. (1994).

Architectural alterations in rat cerebral microvessels after hypobaric hypoxia. *Brain Research, 660*(1), 73-80.

Moraska, A., Deak, T., Spencer, R. L., Roth, D., & Fleshner, M. (2000). Treadmill running produces both positive and negative physiological adaptations in Sprague-Dawley rats. American *Journal of Physiology: Regulatory, Integrative, and Comparative Physiology, 279*, R1321-R1329.

Mouton, P. R. (2002). *Principles and Practices of Unbiased Stereology: An Introduction for Bioscientists*. Baltimore: The Johns Hopkins University Press.

Mu, D., Jiang, X., Sheldon, R.A., Fox, C. K., Hamrick, S. E. G., Vexler, Z. S., & Ferriero, D. M. (2003). Regulation of hypoxia-inducible factor 1α and induction of vascular endothelial growth factor in a rat neonatal stroke model. *Neurobiology of Disease, 14*, 524-534. doi: 10.1016/j.nbd.2003.08.020

Mu, J. S., Li, W. P., Yao, Z. B., & Zhou, X. F. (1999). Deprivation of endogenous brain -derived neurotrophic factor results in impairment of spatial learning and memory in adult rats. *Brain Research, 835*, 259-265.

Murre, C., Bain, G., van Dijk, M. A., Engel, I., Furnari, B. A., Massari, M. E.,…Stuiver, M. H. (1994). Structure and function of helix-loop-helix proteins. *Biochimica et Biophysica Acta, 1218*, 129-135.

Ndubuizu, O. I., Chavez, C. P., and LaManna, J. C. (2009). Increased prolyl 4-hyroxylase expression and differential regulation of hypoxia-inducible factors in the aged rat brain. *American Journal of Physiology: Regulatory, Integrative, and Comparative Physiology, 297*, R158-R165. doi: 10.1152/ajpregu.90829.2008

Ndubuizu, O. I., Tsipis, C. P., Li, A., & LaManna, J. C. (2010). Hypoxia-inducible

factor-1 (HIF-1)-independent microvascular angiogenesis in the aged rat brain. *Brain Research, 1366*, 101-109. doi: 10.1016/j.brainres.2010.09.064

Neeper , S. A., Gomez-Pinilla, F., Choi, J., & Cotman, C. W. (1996). Physical activity increases mRNA for brain derived neurotrophic factor and nerve growth factor in rat brain. *Brain Research, 726*, 49-56.

Newrzella, D., Pahlavan, P. S., Kruger, C., Boehm, C., Sorgenfrei, O., Schrock, H.,…Schneider, A. (2007). The functional genome of CA1 and CA3 neurons under native conditions and in response to ischemia. *BMC Genomics, 8*, 370-387. Retrieved from http://www.biomedcentral.com/1471-2164/8/370

Nitatori, T., Sato, N., Waguri, S., Karasawa, Y., Araki, H., Shibanai, K., Kominami, E., & Uchiyama, Y. (1995). Delayed neuronal death in the CA1 pyramidal cell layer of the gerbil hippocampus following transient ischemia is apoptosis. *Journal of Neuroscience, 15*(2), 1001-1011.

O'Callaghan, R. M., Griffin, E. W., & Kelly, A. M. (2009). Long-term treadmill exposure protects against age-related neurodegenerative change in rat hippocampus. *Hippocampus, 19*, 1019-1029. doi: 10.1002/hipo.20591

O'Callaghan, R. M., Ohle, R., & Kelly, A. M. (2007). The effects of forced exercise on hippocampal plasticity in the rat: A comparison of LTP and spatial- and non-spatial learning. *Behavioural Brain Research, 176*, 362-366. doi: 10.1016/j.bbr.2006.10.018

Ogle, M. E., Gu, X., Espinera, A. R., Wei, L. (2012). Inhibition of prolyl hydroxylases by

dimethyloxaloylglycine after stroke reduces ischemic brain injury and requires hypoxia-inducible factor-1α. *Neurobiology of Disease, 45*, 733-742. doi: 10.1016/j.nbd.2011.10.020

Olfert, I. M., Breen, E. C., Mathieu-Costello, O., & Wagner, P. D. (2001). Skeletal muscle capillarity and angiogenic mRNA levels after exercise training in normoxia and chronic hypoxia. *Journal of Applied Physiology, 91*, 1176-1184.

Olfert, I. M., Howlett, R. A., Tang, K., Dalton, N. D., Gu, Y., Peterson, P. L., Wagner, P. D., & Breen, E. C. (2009). Muscle-specific VEGF deficiency greatly reduces exercise endurance in mice. *Journal of Physiology, 587*(8), 1755-1767. doi: 10.1113/jphysiol.2008.164384

Olfert, I. M., Howlett, R. A., Wagner, P.D. &Breen, E. C. (2010). Myocyte vascular endothelial growth factor is required for exercise-induced skeletal muscle angiogenesis. *American Journal of Physiology: Regulatory, Integrative, and Comparative Physiology, 299*, R1059-R1067. doi: 10.1152/ajpregu.00347.2010

Olson, A. L., & Pessin, J. E. (1996). Structure, function, and regulation of the mammalian facilitative glucose transporter gene family. *Annual Review of Nutrition, 16*, 235-256.

O'Rourke, J. F., Tian, Y., Ratcliffe, P. J., & Pugh, C. W. (1999). Oxygen-regulated and transactivating domains in endothelial PAS protein 1: Comparison with hypoxia-inducible factor-1α. *Journal of Biological Chemistry, 274*(4), 2060-2071.

Pallant, J. (2004). SPSS survival manual: A step by step guide to data analysis using SPSS (2nd ed.). Chicago: Open University Press.

Park, E., Bell, J. D., Siddiq, I. P., & Baker, A. J. (2009). An analysis of regional microvascular loss and recovery following two grades of fluid percussion trauma: A role for hypoxia-inducible factors in traumatic brain injury. *Journal of Cerebral Blood Flow and Metabolism, 29*, 575-584. doi: 10.1038/jcbfm.2008.151

Pereira, A. C., Huddleston, D. E., Brickman, A. M., Sosunov, A. A., Hen, R., McKhann, G. M.,…Small, S. A. (2007). An *in vivo* correlate of exercise-induced neurogenesis in the adult dentate gyrus. *Proceedings of the National Academy of Sciences of the USA, 104*(13), 5638-5643. doi: 10.1073/pnas.0611721104

Peters, K. G., de Vries, C., & Williams, L. T. (1993). Vascular endothelial growth factor receptor expression during embryogenesis and tissue repair suggests a role in endothelial differentiation and blood vessel growth. *Proceedings of the National Academy of Sciences of the United States of America, 90*, 8915-8919.

Phaneuf, S., & Leeuwenburgh, C. (2000). Apoptosis and exercise. *Medicine & Science in Sports & Exercise, 33*(3), 393-396.

Plate, K. H., Breier, G., Weich, H. A., Mennel, H. D., & Risau, W. (1994). Vascular endothelial growth factor and glioma angiogenesis: Coordiante induction of VEGF receptors, distribution of VEGF protein and possible *in vivo* regulatory mechanisms. *International Journal of Cancer, 59*, 520-529.

Plate, K. H., Breier, G., Weich, H. A., & Risau, W. (1992). Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas *in vivo*. *Nature, 359*, 845-8480.

Pollock, M., Phaneuf, S., Dirks, A., & Leeuwenburgh, C. (2002). The role of apoptosis in

the normal aging brain, skeletal muscle, and heart. *Annals of the New York Academy of Sciences, 959*, 93-107.

Prakash, R. S., Voss, M. W., Erickson, K. I., Lewis, J. M., Chaddock, L., Malkowski, E., Alves, H.,…Kramer, A. F. (2011). Cardiorespiratory fitness and attentional control in aging brain. *Frontiers in Human Neuroscience, 4*, 1-12. doi: 10.3389/fnhum.2010.00229

Pugh, G. W., & Ratcliffe, P. J. (2003). Regulation of angiogenesis by hypoxia: Role of the HIF system. *Nature Medicine, 9*(6), 677-684.

Purves, D., Augustine, G. J., Fitzpatrick, D., Katz, L. C., LeMantia, J., McNamara, J. O., & Williams, S…M. *Neuroscience* (2nd edition). Sunderland, MA: Sinauer Associates, Inc.

Rabie, T., Kunze, R., & Marti, H. H. (2011). Imparied hypoxic response in senescent mouse brain. *International Journal of Developmental Neuroscience, 29*, 655-661.

Radak, Z., Toldy, A., Szabo, Z., Siamilis, S., Nyakas, C., Silye, G., Jakas, J., & Goto, S. (2006). The effects of training and detraining on memory, neurotrophins and oxidative stress markers in rat brain. *Neurochemistry International, 49*, 387-392. doi: 10.1016/j.neuint.2006.02.004

Rahman, M. S., & Thomas, P. (2007). Molecular cloning, characterization and expression of two hypoxia-inducible factor alpha subunits, HIF-1α and HIF-2α, in a hypoxia-tolerant marine teleost, Atlantic croaker (*Micropogonias undulates*). *Gene, 396*, 273-282.

Rankin, E. B., Rha, J., Unger, T. L., Wu, C. H., Shutt, H, P., Johnson, R. S.,…Hasse, V.

H. (2008). Hypoxia-inducible factor-2 regulates vascular tumorigenesis in mice. *Oncogene, 27*, 5354-5358

Rasband, W. S., (1997-2012). ImageJ. *U.S. National Institutes of Health, Bethesda, Marlyand, USA*. Retrieved from http://rsbweb.nih.gov

Rasmussen, P., Brassard, P., Adser, H., Pedersen, M. V., Leick, L., Hart, E., Secher, N. H.,…Pilegaard, H. (2009). Evidence for a release of brain-derived neurotrophic factor from the brain during exercise. *Experimental Physiology, 94*(10), 1062-1069. doi: 10.1113/expphysiol.2009.048512

Rhodes, J. S., van Praag, H., Jeffrey, S., Girard, I., Mitchell, G. S., Garland, T., & Gage, F. H. (2003). Exercise increases hippocampal neurogenesis to high levels but does not improve spatial learning in mice bred for increased voluntary wheel running. *Behavioral Neuroscience, 117*(5), 1006-1016. doi: 10.1037/0735-7044.117.5.1006

Rhyu, I. J., Bytheway, J. A., Kohler, S. J., Lange, H., Lee, K. J., Boklewski, J., McCormick, K.,…Cameron, J. L. (2010). Effects of aerobic exercise training on cognitive function and cortical vascularity in monkeys. *Neuroscience, 167*, 1239-1248. doi: 10.1016/j.neuroscience.2010.03.030

Ribatti, D., Vacca, A., Roccaro, A. M., Crivellato, E., & Presta, M. (2003). Erythropoietin as an angiogenic factor. *European Journal of Clinical Investigation, 33*(10), 891-896.

Richardson, R. S., Wagner, H., Mudaliar, S. R. D., Henry, R., Noyszewski, E. A., &

Wagner, P. D. (1999). Human VEGF gene expression in skeletal muscle: effect of acute normoxic and hypoxic exercise. *American Journal of Physiology: Heart and Circulatory Physiology, 277*, 2247-2252.

Richardson, R. S., Wagner, H., Mudaliar, S. R. D., Saucedo, E., Henry, R., & Wagner, P. D. (2000). Exercise adaptation attenuates VEGF gene expression in human skeletal muscle. *American Journal of Physiology: Heart and Circulatory Physiology, 279*, H772-H778.

Risau, W. (1997). Mechanisms of angiogenesis. *Nature, 386*, 671-674.

Rossi, C., Angelucci, A., Costantin, L., Braschi, C., Mazzantini, M., Babbini, F., Fabbri, M. E.,…Caleo, M. (2006). Brain-derived neurotrophic factor (BDNF) is required for the enhancement of hippocampal neurogenesis following environmental enrichment. *European Journal of Neuroscience, 24*, 1850-1856. doi: 10.1111/j.1460-9568.2006.05059.x

Rowan, R. A., & Maxwell, D. S. (1981). Patterns of vascular sprouting in the postnatal development of the cerebral cortex of the rat. *The American Journal of Anatomy, 160*, 247-255

Schneider, C., Krischke, G., Keller, S., Walkinshaw, G., Arend, M., Rascher, W., Gassmann, M., & Trollmann, R. (2009). Short-term effects of pharmacologic HIF stabilization on vasoactive and cytotrophic factors in developing mouse brain. *Brain Research, 1280*, 43-51. doi: 10.1016/j.brainres.2009.05.023

Semenza, G.L. (2000a). HIF-1: mediator of physiological and pathophysiological responses to hypoxia. *Journal of Applied Physiology, 88*, 1474-1480.

Semenza, G.L. (2000b). Surviving ischemia: adaptive responses mediated by hypoxia

-inducible factor 1. *Journal of Clinical Investigation, 106*(7), 809-812.

Seo, K., Choi, J., Park, M., & Rhee, C. (2001). Angiogenesis effects of nerve growth factor (NGF) on rat corneas. *Journal of Veterinary Science, 2*(2), 125-130.

Shalaby, F., Rossant, J., Yamaguchi, T. P., Gertsenstein, M., Wu, X., Breitman, M. L., Schuh, A.C. (1995). Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature, 376*, 62-66.

Shao, G., Gao, C., & Lu, G. (2005). Alterations of hypoxia-inducible factor-1 alpha in the hippocampus of mice acutely and repeatedly exposed to hypoxia. *Neurosignals, 14*, 255-261. doi:10.1159/000088641

Sharp, F. R., & Bernaudin, M. (2004). HIF1 and oxygen sensing in the brain. *Nature Reviews Neuroscience, 5*, 437-448. doi: 10.1038/nrn1408

Shibuya, M. (2006). Differential roles of vascular endothelial growth factor receptor-1 and receptor-2 in angiogenesis. *Biochemistry and Molecular Biology, 39*(5), 469-478.

Shors, T. J., Townsend, D. A., Zhao, M., Kozorovitskiy, Y., & Gould, E. (2002). Neurogenesis may relate to some but not all types of hippocampal-dependent learning. *Hippocampus, 12*, 578-584. doi: 10.1002/hipo.10103

Shweiki, D., Itin, A., Soffer, D., & Keshet, E. (1992). Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-mediated angiogenesis. *Nature, 359*, 843-845.

Siddiq, A., Aminova, L. R., & Ratan, R. R. (2007). Hypoxia inducible factor prolyl 4

-hyroxylase enzymes: Center stage in the battle against hypoxia, metabolic compromise, and oxidative stress. Neurochemical Research, 32, 931-946. doi: 10.1007/s11064-006-9268-7

Sikorski, A. M., Hebert, N., & Swain, R. A. (2008). Conjugated linoleic acid (CLA) inhibits new vessel growth in the mammalian brain. *Brain Research, 1213*, 35-40. doi: 10.1016/j.brainres.2008.01.096

Sim, Y., Kim, S., Kim, J., Yoon, S., Kim, S. S., Chang, H., Lee, T.,…Kim, C. J. (2005). Long-term treadmill exercise overcomes ischemia-induced apoptotic neuronal death in gerbils. *Physiology & Behavior, 84*, 733-738. doi: 10.1016/j.physbeh.2005.02.019

Soldani, P., Gesi, M., Lenzi, P., Natale, G., Fornal, F., Pellegrini, A., Ricciardi, M. P., Paparelli, A. (1999). Long-term exposure to noise modifies rat adrenal cortex ultrastructure and corticosterone plasma levels. *Journal of Submicroscopic Cytology and Pathology, 31*(3), 441-448.

Sowter, H. M., Raval, R., Moore, J., Ratcliffe, P. J., & Harris, A. L. (2003). Predominant role of hypoxia-inducible transcription factor (Hif)-1α versus Hif-2α in regulation of the transcriptional response to hypoxia. *Cancer Research, 63*, 6130-6134.

Stranahan, A. M., Khalil, D., & Gould, E. (2006). Running induced widespread structural alterations in the hippocampus and entorhinal cortex. *Hippocampus, 17*, 1017-1022. doi: 10.1002/hipo.20348

Stroka, D.M., Burkhardt, T., Desbaillets, I., Wenger, R.H., Neil, D.A.H., Bauer,

C.,…Candinas, D. (2001). HIF-1 is expressed in normoxic tissue and displays an organ-specific regulation under systemic hypoxia. *FASEB Journal, 15*, 2445-2453.

Sun, Y., Jin, K., Xie, L., Childs, J., Mao, X. O., Logvinova, A., & Greenberg, D. A. (2003). VEGF-induced neuroprotection , neurogenesis, and angiogenesis after focal cerebral ischemia. *Journal of Clinical Investigation, 111*, 1843-1851. doi: 10.1172/JCI200317977

Suri, C., Jones, P. F., Patan, S., Bartunkova, S., Maisonpierre, P. C., Davis, S., Sato, T. N., Yancopoulos, G. D. (1996). Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell, 87*, 1171-1180.

Svensson, A., Azarbayjani, F., Backman, U., Matsumoto, T., & Christofferson, R. (2005). Digoxin inhibits neuroblastoma tumor growth in mice. *Anticancer Research, 25*, 207-212.

Swain, R. A., Harris, A. B., Wiener, E. C., Dutka, M. V., Morris, H. D., Theien, B. E.,…Greenough, W. T. (2003). Prolonged exercise induced angiogenesis and increases cerebral blood volume in primary motor cortex of the rat. *Neuroscience, 117*, 1037-1046. doi: 10.1016/S0306-4522(02)00664-4

Szatkowski, M., & Atwell, D. (1994). Triggering and execution of neuronal death in brain ischaemia: Two phases of glutamate release by different mechanisms. *TINS, 17*(9), 359-365.

Taie, S., Ono, J., Iwanaga, Y., Tomita, S., Asaga, T., Chujo, K., & Ueki, M. (2009). Hypoxia-inducible factor-1α has a key role in hypoxic preconditioning. *Journal of Clinical Neuroscience, 16*, 1055-1060. doi: 10.1016/j.jocn.2008.09.024

Takagi, K., Ginsberg, M. D., Globus, M. Y. T., Dietrich, W. D., Martinez, E., Kraydieh, S., Busto, R. (1993). Changes in amino acid neurotransmitters and cerebral blood flow in the ischemic penumbral region following cerebral middle artery occlusion in rats: Correlation with histopathology. *Journal of Cerebral Blood Flow and Metabolism, 13*, 575-585.

Talks, K. L., Turley, H., Gatter, K. C., Maxwell, P. H., Pugh, C. W., Ratcliffe, P. J., & Harris, A. L. (2000). The expression and distribution of the hypoxia-inducible factors HIF-1α and HIF-2α in normal human tissues, cancers, and tumor-associated macrophages. *American Journal of Pathology, 157*(2), 411-421.

Tang, K., Xia, F. C., Wagner, P. D., & Breen, E. C. (2010). Exercise-induced VEGF transcriptional activation in brain, lung, and skeletal muscle. *Respiratory Physiology & Neurobiology, 170*, 16-22.

Thompson, K. J., Bulinski, S. C., Powell, S. K., Sikorski, A. M., & Swain, R. A. (2000). *Time-dependent expression of the tyrosine kinase receptors FLK-1 and FLT-1 in the cerebellum of the exercised rat*. Poster session presented at the Society for Neuroscience, New Orleans.

Tian, H., McKnight, S. L., & Russell, D. W. (1997). Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. *Genes & Development, 11*, 72-82.

Toscano-Silva, M., da Silva, S. G., Scorza, F. A., Bonvent, J. J., Cavalheiro, E. A., & Arida, R. M. (2010). Hippocampal mossy fiber sprouting induced by forced and voluntary physical exercise. *Physiology & Behavior, 101*, 302-308. doi: 10.1016/j.physbeh.2010.05.012

Trejo, J. L., Carro, E., & Torres-Aleman, I. (2001). Circulating insulin-like growth factor I mediates exercise-induced increases in the number of new neurons in the adult hippocampus. *The Journal of Neuroscience, 21*(5), 1628-1634.

Urbaniak, G. C., & Plous, S. (2011). Research Randomizer (Version 3.0). Retrieved from http://www.randomizer.org/

Uysal, N., Tugyan, K., Kayatekin, B. M., Acikgoz, O., Bagriyanik, H. A., Gonenc, S., Ozdemir, D.,…Semin, I. (2005). The effects of regular exercise in adolescent period on hippocampal neuron density, apoptosis and spatial memory. *Neuroscience Letters, 383*, 241-245. doi: 10.1016/j.neulet.2005.04.054

Van der Borght, K., Kobor-Nyakas, D. E., Klauke, K., Eggen, B. J. L., Nyakas, C., Van der Zee, E. A., & Meerlo, P. (2009). Physical exercise leads to rapid adaptations in hippocampal vasculature: Temporal dynamics and relationship to cell proliferation and neurogenesis. *Hippocampus, 19*, 928-936. doi: 10.1002/hipo.20545.

van Praag, H., Kempermann, G., & Gage, F. H. (1999). Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. *Nature Neuroscience, 2*(3), 266-270. Retrieved from http://neurosci.nature.com

van Praag, H., Kempermann, G., & Gage, F. H. (2000). Neural consequences of environmental enrichment. *Nature Reviews Neuroscience, 1*, 191-198.

van Praag, H., Shubert, T., Zhao, C., & Gage, F. H. (2005). Exercise enhances learning and hippocampal neurogenesis in aged mice. *Journal of Neuroscience, 25*(38), 8680-8685. doi: 10.1523/jneurosci.1731-05.2005

Vaynman, S., Ying, Z., & Gomez-Pinilla, F. (2004). Hippocampal BDNF mediates the

efficacy of exercise on synaptic plasticity and cognition. *European Journal of Neuroscience, 20*, 2580-2590. doi: 10.1111/j.1460-9568.2004.03720.x

Wang, G. L., Jiang, B., Rue, E. A., & Semenza, G. L. (1995). Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated cellular O_2 tension. *Proceedings of the National Academy of Sciences of the United States of America, 92*, 5510-5514.

Ward, N. L., & LaManna, J. C. (2004). The neurovascular unit and its growth factors: coordinated response in the vascular nervous system. *Neurological Research, 26*, 870-883. doi: 10.1179/016164104X3798

Ward, N. L., Moore, E., Noon, K., Spassil, N., Keenan, E., Ivanco, T. L., & LaManna, J. C. (2007). Cerebral angiogenic factors, angiogenesis, and physiological response to hypoxia differ among four commonly used mouse strains. *Journal of Applied Physiology, 102*, 1927-1935. doi: 10.1152/japplphysiol.00909.2006

Weisener, M. S., Turley, H., Allen, W. E., William, C., Eckardt, K. U., Talks, K. L.,…Maxwell, P. H. (1998). Induction of endothelial PAS domain protein-1 by hypoxia: Characterization and comparison with hypoxia-inducible factor-1α. *Blood, 92*(7), 2260-2268.

Wenger, R. H., Kvietikova, I., Rolfs, A., Gassmann, M., & Marti, H. H. (1997). Hypoxia -inducible factor-1α is regulated at the post-mRNA level. *Kidney International, 51*, 560-593.

Wiener, C. M., Booth, G., & Semenza, G. L. (1996). In vivo expression of mRNAs encoding hypoxia-inducible factor 1. *Biochemical and Biophysical Research Communications, 225*, 485-488.

Wilson, R. S., Mendes de Leon, C. F., Barnes, L. L., Schneider, J. A., Bienias, J. L., Evans, D. A., & Bennett, D. A. (2002). Participation in cognitively stimulating activities and risk of incident Alzheimer disease. *Journal of the American Medical Association, 287*(6), 742-748.

Winter, B., Breitenstein, C., Mooren, F. C., Voelker, K., Fobker, M., Lechterman, A., Krueger, K.,…Knecht, S. (2007). High impact running improves learning. *Neurobiology of Learning and Memory, 87*, 597-609. doi: 10.1016/j.nlm.2006.11.003

Won, S. J., Kim, D. Y., & Gwag, B. J. (2002). Cellular and molecular pathways of ischemic neuronal death. *Journal of Biochemistry and Molecular Biology, 35*(1), 67-86.

Wyllie, A. H. (1997). Apoptosis: An overview. *British Medical Bulletin, 53*(3), 451-465.

Yancopoulos, G. D., Davis, S., Gale, N. W., Rudge, J. S., Wiegand, S. J., & Holash, J. (2000). Vascular-specific growth factors and blood vessel formation. *Nature, 407*, 242-248.

Yang, S. P., Bergo, G. W., Krasney, E., & Krasney, J. A. (1994). Cerebral pressure-flow and metabolic responses to sustained hypoxia: Effect of CO2. *Journal of Applied Physiology, 76*(1), 303-313.

Yenari, M. A., Liu, J., Zheng, Z., Vexler, Z. S., Lee, J. E., Giffard, R. G. (2005). Antiapoptotic and anti-inflammatory mechanisms of heat shock protein protection. *Annals of the New York Academy of Sciences, 1053*, 74-83. doi: 10.1196/annals.1344.007

Yoshida, T., Zhang, H., Iwase, T., Shen, J., Semenza, G. L., & Campochiaro, P. A.

Digoxin inhibits retinal ischemia-induced HIF-1α expression and ocular neovascularization. *The FASEB Journal,* 24, 1759-1767.

Zhang, Q., Li, X., Cui, X., & Zuo, P. (2005). D-Galactose injured neurogenesis in the hippocampus of adult mice. *Neurologic Research, 27*, 552-556. doi: 10.1179/016164105X25126

Zhang, H., Qian, D. Z., Tan, Y. S., Lee, K., Gao, P., Ren, Y. R.,…Semenza, G. L. (2008). Digoxin and other cardiac glycosides inhibit HIF-1α synthesis and block tumor growth. *PNAS, 105*(50), 10.1073/pnas.0809763105

Zhang, L., Qu, Y., Yang, C., Tang, J., Zhang, X., Mao, M., Mu, D., & Ferriero, D. (2009). Signaling pathway involved in hypoxia-inducible factor-1α regulation in hypoxic-ischemic cortical neurons in vitro. *Neuroscience Letters, 461*, 1-6. doi: 10.1016/j.neulet.2009.03.091

Zhu, S., Pham, T., Aberg, E., Brene, S., Winblad, B., Mohammed, A. H., & Baumans, V. (2006).Neurotrophin levels and behaviour in BALB/c mice: Impact of intermittent exposure to individual housing and wheel running. *Behaviroural Brain Research, 167*, 1-8. doi: 10.1016/j.bbr.2005.02.038

CURRICULUM VITAE

Kiersten L. Berggren

Education:

2010-Present Ph.D. candidate, University of Wisconsin – Milwaukee, Department of Psychology (emphasis: behavioral neuroscience). Graduation: August 2013.

2008-2010 Master of Sciences, Forensic Toxicology, Beth-El School of Nursing and Health Sciences, University of Colorado – Colorado Springs (UCCS). Master's thesis (May 15, 2010): "*Postmortem Redistribution and Cocaine Concentrations in Blood and Brain Tissue*"

2003-2008 Master of Science in Psychology (emphasis: behavioral neuroscience), University of Wisconsin-Milwaukee. Master's thesis (December 19, 2005): *"Regional Angiogenesis and Angiogenesis Persistence in the Motor Cortex in the Exercising Rat."*

1999-2002 Bachelor of Arts, Psychology and Sociology. University of Colorado-Boulder Graduation date: May 2002.

Research:

2011 Research consultant for collaborative work with PhysioGenix, Inc., conducted at the University of Wisconsin-Milwaukee. Collaborator – Jennifer Tinklenberg

2010-Present Doctoral dissertation research at the University of Wisconsin – Milwaukee. Dissertation advisor – Dr. Rodney Swain

2008-2010 Master's research at the University of Colorado at Colorado Springs. Master's advisor - David Swaby

2003-2008 Master's research, dissertation pilot research, and various other research projects at the University of Wisconsin - Milwaukee. Master's and dissertation advisor – Dr. Rodney Swain

2006-2008 Research collaboration with PhysioGenix, Inc. (research regarding consomic animals and behavior), University of Wisconsin – Milwaukee

2004 Research consultant for MPRES, Inc. Duties included the organization, writing, distribution, and analysis of results of a regional survey/questionnaire of medical professionals in women's health.

2001 Research assistant (supervisor: Dr. Kent Hutchison), University of Colorado – Boulder

Professional Conference Presentations:

2013 Berggren, K.L., Kay, J.J.M., Pochinski, B.R., Hoell, A.R., & Swain, R.A. (2013). Voluntary exercise of manipulation of HIF-1α expression increases hippocampal capillary density and improves MWM performance in adult rats.

2012 Berggren, K.L., Ahuja, B.A., Kay, J.J.M., Pochinski, B.R., & Swain, R.A. (2012). Treadmill exercise rapidly induces the expression of HIF-1α in the hippocampus of the adult rat. Society for Neuroscience annual meeting.

2012 Berggren, K.L. (2012). Exercise induces hypoxia in the hippocampus of the adult rat. University of Wisconsin-Milwaukee Psychology Graduate Student Symposium.

2012 Ahuja, B.A., Berggren, K.L., & Swain, R.A. (2012). Exercise and hypoxia in the rat hippocampus. Chicago Chapter of the Society for Neuroscience annual meeting.

2008 Berggren, K.L. (2008). Exercise-induced angiogenesis in the CNS of Dahl salt -sensitive and SSBN.13 consomic rats. University of Wisconsin-Milwaukee Psychology Graduate Student Symposium

2008 Berggren, K., Kerr, A., Wolter, M., Koenigs, K., Jarome, T., Nye, S., Helmstetter, F., & Swain, R. (2008). Impaired learning and behavioral adaptation in the SSBN.13 consomic rat. Milwaukee Chapter of Society for Neuroscience annual meeting.

2008 Kerr, A., Jarome, T., Berggren, K., Wolter, M., Koenigs, K., Nye, S., Helmstetter, F., & Swain, R. (2008). Enhanced learning and behavioral adaptation in the FHH.BN1 consomic rat. Milwaukee Chapter of Society for Neuroscience annual meeting.

2008 Nye, S.H., Kerr, A.L., Berggren, K.L., Jacob, H.J., Cook, J.M., and Swain, R.A. (2008). Rat strains with natural deficits in cognition for accelerating drug development. Society for Neuroscience annual meeting.

2008 Berggren, K.L., Kerr, A.L., Iles, B.W., Nye, S.H., and Swain, R.A. (2008). Exercise-induced angiogenesis in the CNS of Dahl salt-sensitive and SSBN.13 consomic rats. Society for Neuroscience annual meeting.

2007 Nye, S., Wolter, M., Kerr, A., Koenigs, K., Berggren, K., Jarome, T., Helmstetter, F., and Swain, R. (2007). Chromosomal substitution strains of rat for studying human psychological disorders. Society for Neuroscience annual meeting

2007 Kerr, A., Jarome, T., Berggren, K., Wolter, M., Koenigs, K., Nye, S., Helmstetter, F., and Swain, R. (2007). Enhanced learning and behavioral adaptation in the FHH.BN1 consomic rat. Society for Neuroscience annual meeting.

2007 Berggren, K., Kerr, A., Wolter, M., Koenigs, K., Jarome, T., Nye, S., Helmstetter, F., and Swain, R. (2007). Impaired learning and behavioral adaptation in the SSBN.13 consomic rat. Society for Neuroscience annual meeting.

2005 Berggren, K.L., Sikorski, A.M., & Swain, R.A. (2005). Exercise-induced angiogenesis in rat motor cortex does not persist following a prolonged period of inactivity. Society for Neuroscience annual convention.

2004 Berggren, K.L. (2004). Pre- and post-ischemic exercise and neuroprotection in rats. University of Wisconsin-Milwaukee Psychology Graduate Student Symposium.

Publications:

Swain, R.A., Berggren, K.L., Kerr, A.L., Patel, A., Peplinski, C., & Sikorski, A.M. (2012). On aerobic exercise and behavioral and neural plasticity. *Brain Sciences, 2*, 709-744. doi: 10.3390/brainsci2040709

Berggren, K.L., Ahuja, B.A., Kay, J.J.M., Pochinski, B.R., Hoell, A.R., & Swain, R.A. *Treadmill exercise rapidly induces the expression of hypoxia-inducible factor-1alpha in the hippocampus of the adult rat.* Submitted manuscript.

Berggren, K.L., Kay, J.J.M., & Swain, R. A. *Examining cerebral angiogenesis in response to physical exercise.* Submittted manuscript.

Teaching Experience:

2011-Present Associate Lecturer – Advanced Physiological Psychology, University of Wisconsin-Milwaukee

2010-Present Teaching assistant – University of Wisconsin-Milwaukee. Courses: Research Methods in Psychology, Cognitive Processes

2009-2010 Writing Center Tutor and APA Workshop Instructor, University of Colorado – Colorado Springs Excel Writing Center. Supervisor: Dr. Traci Freeman

2007 Lecturer – Physiological Psychology; University of Wisconsin – Milwaukee

2006 Development team member for the online Introduction to Psychology (UPACE) course; Supervisor – Dr. Diane Reddy, University of Wisconsin – Milwaukee

2003-2008 Teaching assistant – University of Wisconsin-Milwaukee (courses: Research Methods, Cognitive Processes, Introduction to Psychology, Physiological Psychology, Social Psychology)

Honors/Awards

2012: Second place award – AGSIP Graduate Student Research Symposium, University of Wisconsin – Milwaukee

2010: Sigma-Xi Grants in Aid of Research recipient

2010: Beth-El College of Nursing and Health Sciences Forensic Science Student of the Year Award

2009-2010: UCCS Graduate Student Fellowship

2009-2010: CU Retired Faculty Association (CURFA) Award
2008-2009: UCCS Chancellor's Scholarship

2008: Second place award – AGSIP Graduate Student Research Symposium, University of Wisconsin – Milwaukee.

2004-2005: UWM Chancellor's Fellowship

2004: Speaking award, second place- AGSIP Graduate Student Research Symposium, University of Wisconsin-Milwaukee.

2003-2004: UWM Chancellor's Fellowship

Professional Society Memberships:
2010-present: Student member - Society for Neuroscience, American Psychological Association

2009- 2010: Student member - American Academy of Forensic Sciences

2008-present: Student member - American Association for the Advancement of Science

2003-2008: Student member - Society for Neuroscience, American Psychological Association

2007-2008: Student member - American Physiological Society